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THE EFFECT OF VASCULAR OCCLUSION ON TRANSPLANTED TUMORS [1]

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INTRODUCTION

Previous work with transparent chamber preparations in the mouse (*1, 2*) has shown that the hemorrhage and cell damage produced in certain transplanted tumors by the injection of a polysaccharide preparation from *Serratia marcescens* is preceded by slowing of the systemic circulation, with accompanying intravascular agglutination of erythrocytes, stasis, and occlusion of the capillaries both within the tumor and in adjacent normal striated muscle. In addition to the circulatory changes described above, several other indices of tumor damage were used. Histologic examination was carried out in several instances, but chief reliance was placed on *in vivo* criteria, as follows: (1) The development of vascular stasis and hemorrhage within the tumor, (2) the later development of opacity of such avascular areas of cells in contrast to the more translucent appearance of unaffected areas, (3) failure to reestablish circulation in affected areas, (4) in cases which were followed for longer than 48 hours, liquefaction of the previously opaque areas and observed disintegration of structure, with ulceration of the underlying skin.

The first criterion of tumor damage has been used by other workers with bacterial products (*3*). In using increased opacity as one index of tumor damage, reference is made to the common use of this phenomenon by workers with living tissues *in vivo* or *in vitro* (*4*).[3] In our experience it has

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[3] Earle, Wilton R.: Personal communication.

been found invariably that areas of cells showing opacity of the type described, developing within the tumor 24 to 48 hours following vascular occlusion, show progressive changes leading to liquefaction necrosis and ulceration. In addition, such areas show the diffuse staining with intravital basic dyes characteristic of cell damage *in vitro* (*5*). It is pointed out that this type of opacity is not to be confused with an accumulation of leukocytes at the periphery of the tumor or local bacterial infection.

In the experiments described above (*1*, *2*) no evidence was found of a direct and specific action of these substances on tumor cells. The hemorrhage and cell damage produced in the tumor seemed to be secondary effects resulting from a tissue anoxia. This tissue anoxia was postulated since slowing of the blood flow, stasis, occlusion of capillaries, and intravascular agglutination of erythrocytes would tend to reduce the efficiency of oxygen transport to the cells of the capillary walls and to the cells of the tumor. The potential role of intravascular agglutination phenomena in the production of tissue anoxia has been considered by several workers (*6*, *7*).

These views on the possible importance of tissue anoxia in tumor damage by various agents are divergent from the conclusions of other workers who state that such injurious substances act in a direct and specific manner on the tumor cells (*8*, *9*).

The experiments reported here are concerned with the effects of local mechanical occlusion of the circulation on normal and malignant tissues. The tumors used were chosen for their varying susceptibilities to the damaging effects of bacterial polysaccharide. The sarcoma responds to this agent by hemorrhage and necrosis of a part of the tumor, as previously reported (*1*), while the mammary tumor shows neither hemorrhage nor evidence of cell damage.[4] In this manner, the role of reduced blood supply and local tissue anoxia in tumor damage could be studied without the introduction of highly toxic chemical agents into the host.

MATERIALS AND METHODS

A method for the direct microscopic observation and measurement of the effects of vascular occlusion on transplanted tumors was provided by the use of the transparent chamber technique in the mouse (*10*). The general procedure was as follows: Tumor tissues were transplanted into transparent chambers in the dorsal skin flap at the time of insertion of the chamber. Quantitative measurements were made of the size of the tumors and the relative amounts of vascular tissues (*11*) prior to and following the production of local vascular occlusion. Photographic records were obtained of significant stages in the changes that occurred.

[4] Youngner, J. S.: Unpublished data.

Local vascular occlusion in the window area was produced by the use of two small metal rods clamped tightly to both sides of the skin flap at the base of the transparent chamber (Fig. 1). Microscopic observations were made to insure that there was a complete stoppage of blood flow in the window area. The clamps were allowed to remain in place for from one-half to 3 hours, then removed, and the chamber was carefully observed microscopically for any changes which might occur, either immediately or within the next 24 to 72 hours.

Histologic examination was carried out in several instances, but the chief criteria of tumor damage used were (1) the development of vascular stasis and hemorrhage, (2) development of grayish opacity in such avascular areas in contrast to the more translucent tissue in unaffected sites, (3) failure to re-establish circulation in affected areas, and (4) in many cases, liquefaction of these previously opaque areas with ulceration of the external skin surface.

The tumors used were the L-sarcoma [5] and a mammary adenocarcinoma (C3HBA), [6] both derived from and transplantable in strain C3H mice. In addition, the reactions of the chamber area to vascular occlusion were tested in control animals which did not bear tumor transplants and in animals which had spontaneously formed granulation tissue present within the chamber.

RESULTS

In control mice which did not bear tumor transplants, vascular occlusion for periods of from 1 to 3 hours produced no observable changes in the tissues comprising the window area (table 1). Neither hemorrhage nor evidence of tissue damage was seen. Blood flow resumed immediately upon removal of the obstruction, and, except for a transitory reactive hyperemia, within a short time the circulatory state and functional vascular levels were normal. Vasomotor activity resumed, and intermittent contraction of striated muscle occurred. Immediately after circulation was resumed, numerous, large, whitish masses were observed in the circulation. These clumps persisted for several minutes, then disappeared. Apparently similar masses have been described by the authors following the intravenous injection into mice of a polysaccharide from *Serratia marcescens* (*2*). The nature of these masses is not known. Possibilities being considered are that these masses may be platelet emboli (*12*) or a product of damage to the lining of blood vessels (*13*).

During the period of return of blood vessels to a functional status, intravascular agglutination of erythrocytes in varying degree was noted in some animals. These circulatory changes were of short duration in the control animals.

[5] Obtained through the courtesy of Dr. W. R. Earle of the National Cancer Institute.
[6] Obtained through the courtesy of Dr. M. K. Barrett of the National Cancer Institute.

TABLE 1.—*Effect of vascular occlusion on normal tissues in control windows*

Experiment No.	Time after insertion of window	Duration of occlusion	Hemorrhage	Evidence of tissue damage
	Days	*Minutes*		
1	27	60	None	None
2	20	95	None	None
3	10	125	None	None
4	11	125	None	None
5	39	130	None	None
6	6	135	None	None
7	7	180	None	None

SARCOMA

When the blood supply to windows bearing growing sarcoma transplants was obstructed for periods of from one half to 3 hours, hemorrhage developed in all tumors in which occlusion lasted longer than one-half hour (table 2). The development of hemorrhage was accompanied by a marked decrease in the number of functional blood vessels in the tumor. The response varied from scattered petechiae (+) to massive hemorrhage (+++), in which case the tumor was obscured by extravasated blood, no other detail being visible. Figure 2 shows the appearance of a sarcoma growing in the window before and 10 minutes after the removal of the vascular occlusion. In almost all cases tumor damage was observed 24 hours later. Figure 3, and Figure 4, illustrate, with fixed and stained material, the changes that occurred in sarcoma L, 24 hours and 3 days, respectively, after release of the tourniquet (3 hours [3] occlusion). In all cases, renewed vascularization and growth of surviving peripheral tissues occurred. Normal tissue surrounding the sarcoma appeared to be unaffected by the treatment, as indicated by the resumption of vasomotor activity and muscle contraction.

It is of interest to note that in the case of experiment No. 10, vascular occlusion of the same tumor was produced on three occasions, at 3-day intervals. The tumor responded each time by hemorrhage and cell damage, although at each succeeding treatment there was less tumor tissue present. Despite the repeated treatment, surviving tissue at the periphery became vascularized and continued to grow.

TABLE 2—*Effect of vascular occlusion on L-sarcoma growing in windows*

Experiment No.	Time after insertion of window	Duration of occlusion	Hemorrhage	Evidence of tissue damage
	Days	*Minutes*		
8	27	30	−	−
9	17	75	+ + +	−
10	19	120	+ + +	+ + +
	22	165	+ + +	+ + +
	25	180	+ + +	+ + +
11	13	145	+	+ +
12	20	150	+ +	+ +
13	13	160	+	−
14	14	180	+ + +	+ + +
15	17	180	+ + +	+ + +

− none.
+ slight.
++ moderate.
+++ severe.

The occurrence of intravascular agglutination phenomena appeared to be somewhat more pronounced following vascular occlusion of sarcoma-bearing windows than that resulting from similar treatment of control windows.

MAMMARY CARCINOMA

In contrast to the reactions of sarcoma to vascular occlusion, mammary carcinomas treated in an identical manner were not affected (table 3). Occasionally, some free red blood cells were observed around the periphery of the tumor, and in some cases there was a significant drop in the functional vascular level in the tumor, but no hemorrhage was seen in the carcinoma tissues. Tumor damage was also absent. Intravascular agglutination phenomena were not so pronounced as in the case of sarcoma.

In several cases not included in the tables, hemorrhage occurred in sarcomas during the period of vascular obstruction or when the clamps were being removed. This was never observed in mammary carcinomas. In fact, in experiment No. 20 (table 3) a sarcoma and carcinoma were implanted into the same window and were growing in close proximity. Before the blood flow was allowed to resume, the sarcoma showed a severe hemorrhage while the mammary carcinoma was unaffected.

TABLE 3.—*Effect of vascular occlusion on mammary carcinoma growing in windows*

Experiment No.	Time after insertion of window	Duration of occlusion	Hemorrhage	Evidence of tissue damage
	Days	*Minutes*		
16	18	70	None	None
	20	120	None	None
17	18	145	None	None
18	23	165	None	None
19	28	165	None	None
20	10	185	None	None

GRANULATION TISSUE

In five windows which had not been prepared specifically for this study, granulation tissue in varying amounts had been formed spontaneously. These windows were also treated in the manner described above, vascular occlusion being applied for periods of from 2 to 3 hours. In every case the blood vessels within granulation tissue responded by severe hemorrhage while the normal tissues comprising the window area remained unaffected (table 4).

TABLE 4.—*Effect of vascular occlusion on granulation tissue present in windows*

Experiment No.	Time after insertion of window	Duration of occlusion	Hemorrhage	Evidence of tissue damage
	Days	*Minutes*		
21	20	115	+ + +	None
3	10	125	+ + +	None
4	11	125	+ + +	None
22	22	170	+ + +	None
23	18	185	+ + +	None

+ + + severe

DISCUSSION

Using the transparent chamber technique, it has been shown that vascular occlusion for periods of 1 to 3 hours produced hemorrhage and cell damage in sarcoma tissues growing in the window, while a mammary carcinoma and normal tissues were unaffected. In this respect, the responses of these tissues to vascular occlusion are identical to their reactions to bacterial polysaccharide under similar conditions.

Previous work of the authors has indicated the importance of host vascular reactions, probably including local tissue anoxia, in the mechanism of action of bacterial polysaccharide. In the present study, mechanical interference with tumor blood supply was used to simulate changes in circulatory supply resulting from injection of the tumor-damaging agents previously studied. The data presented show that mechanical obstruction can *per se* duplicate the action of bacterial polysaccharide in producing hemorrhage and tissue damage in susceptible tumors, while

having no such effect on resistant tissues. It is also of interest to note that granulation tissue in the window area showed a response analogous to that of sarcoma.

From the experiments reported here, it appears likely that hemorrhage and tissue damage within the sarcoma and granulation tissues were probably manifestations of capillary damage due to local tissue anoxia resulting from occlusion of the blood supply. The factors responsible for the different responses of sarcoma and granulation tissue, on the one hand, and mammary carcinoma and normal tissues, on the other, have not been identified. The structure and fragility, permeability, and blood pressure of the capillaries in these two types of tumors should be investigated. The finding that local tissue anoxia can lead to hemorrhage and cellular degenerative changes is supported by the work of others (*14*, *15*).

SUMMARY

Using the transparent chamber technique in C3H mice, it has been found that vascular occlusion for periods of 1 to 3 hours produced hemorrhage and cell damage in growing transplanted sarcoma tissues when blood flow was resumed. Mammary carcinoma and normal tissues treated in a similar manner were unaffected. Granulation tissues showed a response analogous to that of sarcoma.

The relation of these findings to the possible role of local tissue anoxia in the mechanism of action of certain tumor damaging substances is discussed.

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PLATE 81

FIGURE 1.—Transparent chamber in dorsal skin flap of the mouse, showing position and attachment of clamps used to produce vascular occlusion in the window area.

PLATE 82

FIGURE 2.—*A*, Sarcoma before vascular occlusion. *B*, Same tumor as *A*, 10 minutes following release of clamps (duration of occlusion, 75 minutes), showing hemorrhage and reduction of number of functional blood vessels in the tumor. × 50.

A

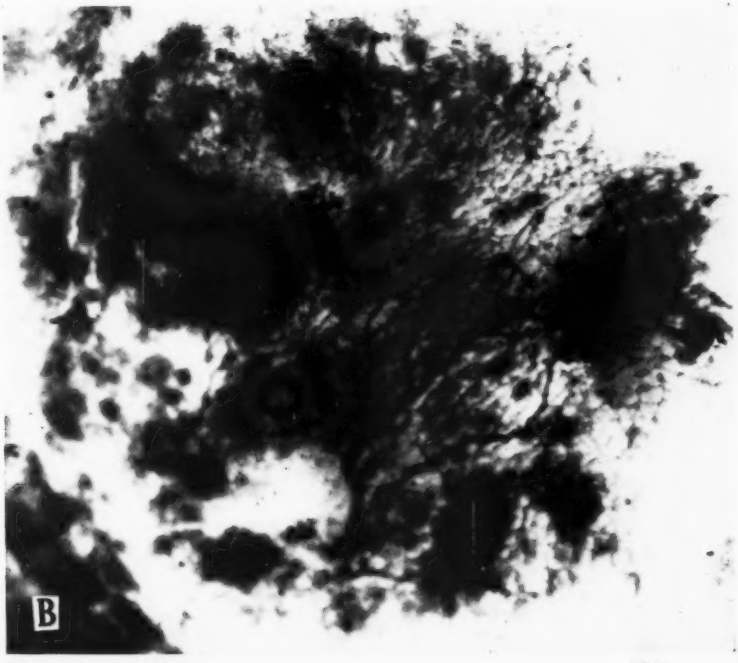
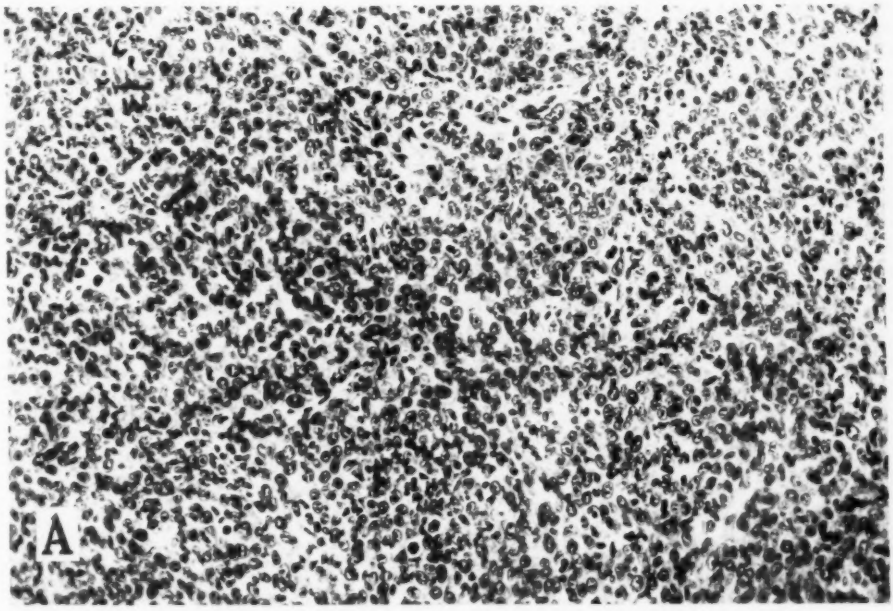

B

PLATE 83

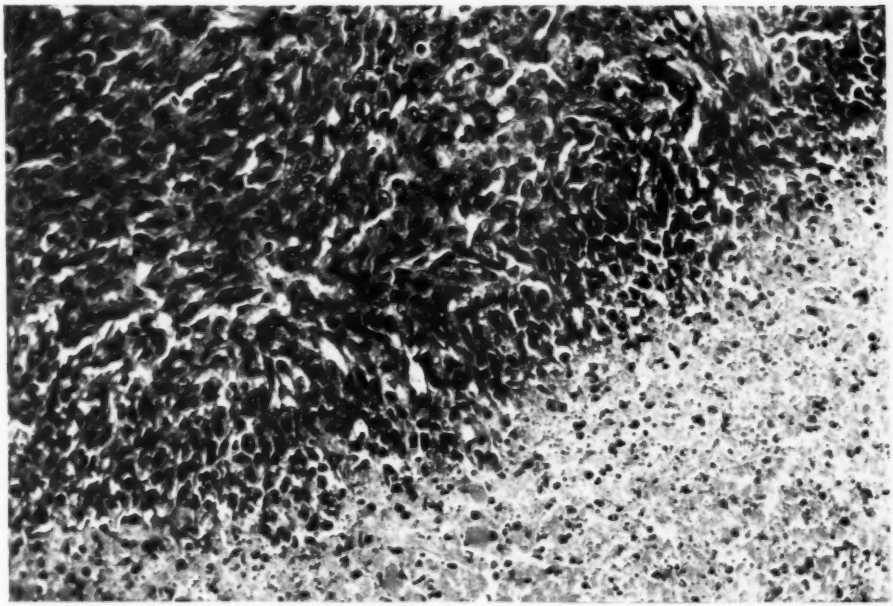
FIGURE 3.—*A*, Sarcoma, untreated. *B*, Sarcoma 24 hours following release of clamps (duration of occlusion, 180 minutes), showing early cell damage; nuclei pyknotic, increased intercellular substance. Zenker-formol fixation, hematoxylin-eosin stain. × 310.


A

B

PLATE 84

FIGURE 4.—Sarcoma 3 days following release of clamps (duration of occlusion, 180 minutes), showing severely damaged area with nuclear fragmentation, cellular destruction, and area of continued cellular proliferation. Zenker-formol fixation, hematoxylin-eosin stain. × 310.



RELATION OF DURATION OF A TRANSPLANTED SARCOMA TO METASTASIS IN THE HAMSTER[1,2,3]

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INTRODUCTION

The occurrence of varied, multiple metastases from subpannicular implants in hamsters which appeared not to be related to the method employed or the condition of the neoplastic material used in transplantation (*1*) and the fact that, in general, investigators do not agree on either the route or the manner of dissemination of sarcoma prompted this investigation. Whether sarcomas are disseminated via the lymphatics, as well as the blood vessels, and whether the active material thus disseminated is composed of aggregates of tumor cells, single cells, or cell-particles varying in size from less than that of the nucleus to submicroscopic proportions, or other cell substances, are questions that have not been answered.

Many pathologists and other investigators hold that sarcoma is disseminated as single cells, or emboli, usually via the blood stream but seldom via the lymphatics (*2–7*). Ewing (*5*) states that lymphatics are comparatively immune to invasion by sarcoma and that "except in lympho-sarcoma, the occurrence of metastases in lymph nodes justifies the suspicion that the tumor is not a sarcoma;" while Boyd (*8*) holds that "embolism by the blood stream is common in both carcinoma and sarcoma." Others (*9–13*) furnish ample justification for the recognition of lymphatics as an important route for dissemination of sarcomas.

MATERIAL AND METHODS

Syrian hamsters, *Mesocricetus auratus*, of both sexes and various ages from a colony now in the sixth year of inbreeding were used in these experiments. The total number of animals used was 391, of which 371

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were subpannicularly implanted, and 20 were used as controls for structure of certain organs.

The 371 subpannicularly implanted hamsters fell into the following age-groups when killed: 85, 50 to 100 days; 100, 101 to 150; 72, 151 to 200; 58, 201 to 250; 37, 251 to 300; and 19, 301 to 400.

The tumor used in this investigation frequently kills the host within 4 weeks. It is a mixed-cell, or polymorphous, sarcoma [4] derived in 1944 by subpannicular injections of 9,10-dimethyl-1,2-benzanthracene and propagated by transplantation (*1*).

The duration of the tumor (days) represents the entire period from implantation to death of the host. Thus, it includes the total period of development of the primary and recurrent tumors, if the implanted tumor was removed one or more times, as well as the period of development of the metastases that may have followed extirpation of the primary, recurrent, or metastatic tumors.

A number of methods of fixing, sectioning, and staining the various organs and structures was employed routinely and for specific purposes. Sections through the thick region and margin of the left anterior and right, or cystic, lobes of the liver of each animal were routinely fixed in sublimate-alcohol and stained with toluidin blue and by the Feulgen process (*14*). Other stains and methods used included Harris', Delafield's, and Heidenhain's hematoxylin with variations and eosin or acid fuchsin, Mallory's triple connective tissue, methylene blue, methyl green, Wright's for smears, and Pappenheim's methyl green-pyronin.

Excision and Transplantation

All steps in removal and transplantation of the tumor were carried out under as nearly aseptic conditions as possible. Great care was taken to avoid unnecessary manipulation of the tumor and incision of the delicate capsule, since it is known that manipulation favors spread and that incision of the capsule favors recurrence (*1*).

Transplantation was effected by injecting scrapings of growing tumor (Solid), aspirated necrotic fluid (Brei), blood from solid tumors (Blood), or scrapings of solid tumor tissue in either blood or brei (Fragments blood or brei) in this work (table 1).

Only fragments of solid, rapidly growing tissues from primary tumors aspirated through an 18-gauge needle into a 2-cc. glass syringe containing a warm modified Locke solution and injected into the lumbar region are now used in routine transplantation. The optimum dose is a few visible fragments of the tumor in 0.5 cc., or less, of the modified Locke solution injected subpannicularly (into the capacious areolar area between the panniculus carnosus muscle and the body wall) in the lumbar region, as described below in method 1. Implantation by this method produced

[4] I believe that this tumor is fundamentally derived from fibroblasts. It appears to be a fibrosarcoma (*6*) with little or no collagen and composed of unorganized, loosely arranged, large and small spindle cells and round cells of various sizes with variable nuclei. Because of these characters and the fact that the growth of the neoplasm in transplantation is very anaplastic I have used the term mixed-cell sarcoma in this paper.

TABLE 1.—*Relation of method of implantation and removal of the tumor to distribution of metastases in axillary and inguinal lymph nodes and lungs in 225 hamsters*

Method of implantation	Duration (days)		No removal of tumor			One removal of tumor			Two removals of tumor			Summary of distribution of metastases				
	Mean	Extremes	Hamsters	Node metastases [1]	Lung metastases	Hamsters	Node metastases [1]	Lung metastases	Hamsters	Node metastases [1]	Lung metastases	Hamsters	Node metastases [1]	Percent	Lung metastases	Percent
			Number			*Number*			*Number*			*Number*				
Solid	31.5	16–54	2	0	0	13	9	0	4	4	2	19	13	68.4	2	10.5
Brei	37.4	15–77	32	7	3	24	9	4	5	2	0	61	18	29.5	7	11.5
Blood	41.2	16–172	36	4	4	25	0	5	2	1	2	63	5	7.9	11	17.4
Fragments + blood brei	29.0	16–61	31	7	1	45	15	11	6	4	1	82	26	31.7	13	15.8
Total	------	------	101	18	8	107	33	20	17	11	5	225	62	27.6	33	14.6

[1] The values under node metastases indicate the number of animals, rather than the number of nodes, involved.

100 percent takes and, when properly placed, the implant seldom invaded the bodywall or ulcerated before attaining a maximum diameter of 25 to 40 mm. Surgical removal of the primary tumor and transplantation was performed at this stage. Thus, the tumor-line was kept relatively free from open lesions and consequent infection.

1. *Injection of solid tumor fragments.* Solid, growing tumor tissue essentially free from blood, necrotic fluid, or other contamination was obtained for transplantation by exposing and scraping a selected subcapsular region of the excised tumor with a sharp scalpel and washing the scrapings off the blade into a warmed sterile vial with a stream of warm salt solution from the hypodermic syringe fitted with an 18-gauge needle, and then aspirating some of the fragments into 0.5 cc. of the Locke solution before inoculating the host.

2. *Injection of necrotic fluid.* Usually an 18-gauge needle was inserted through the skin into soft, nonulcerated tumors, and the desired amount of necrotic fluid (brei) was aspirated into salt solution and injected subpannicularly into the lumbar region of the unanesthetized host. In some instances it was necessary to make a stab-incision into the tumor in order to obtain the necrotic fluid.

3. *Injection of blood from solid tumors.* About 0.25 cc. of blood from an incision made through the skin and deep into the solid tumor was aspirated into an equal amount of the Locke solution and injected subpannicularly into the hamsters.

4. *Fragments + blood or brei.* Scrapings from the tumor in modified Locke solution were aspirated into an equal volume (0.25 cc. or less) of blood from a solid tumor or of brei and injected. Implantation by this method produced 100 percent takes, but the implant had a shorter latent period and a somewhat higher incidence of infection than in method 1.

EXPERIMENTAL PROCEDURE

Implantation Site

The subpannicular, mid-dorsal lumbar region was chosen as the site for routine implantation because of the convenience in examining and removing the tumor and because of its rich supply of blood and lymph vessels.

Lymphatic Drainage

The extent and course of lymphatic drainage of the inoculation site has not been determined satisfactorily. The axillary and inguinal lymph nodes have essentially the same location in the hamster as in the mouse. Although the position and anatomical relations of these to the inoculation site contraindicate such results, the distribution of metastases suggests that these lymph nodes are regional to the inoculation site. The axillary lymph nodes, usually one deep and one (often paired) postaxillary on each side, are situated approximately twice as far from the inoculation site as are the superficial inguinal nodes.

Identification of Metastases

The final identification of metastasis was based upon the results of histologic examination in each case. Since the normal lymph nodes in hamsters are very small, the entire node was usually sectioned. Histologic examination of lymph nodes that were enlarged five or more times always verified the presence of metastasis; however, several nodes which showed no enlargement contained secondary growth.

Pin-point surface and deeper metastases (Fig. 1, *A* and *B*) were readily detected macroscopically in the lungs after fixation in sublimate alcohol. Metastases in the kidneys were detected macroscopically before fixation by the presence of light gray streaks in the ventral side of the organ. Histologically these streaks were found to be areas in various stages of infarction caused by vascular occlusion due to arterial tumor emboli usually in the medullocortical region. The white flecks found in some livers suggested neoplastic growth but proved upon microscopic examination to be accumulations of lymphocytes (*14*).

RESULTS

One third of the 345 tumor-bearing hamsters developed metastases to the regional lymph nodes and/or the lungs. Since only 7.5 percent (26) of these animals developed metastasis without having had the implanted tumor removed, while 25.8 percent (89) that had the tumor removed developed metastasis, it is apparent that the higher rate of metastasis was related to removal of the tumor (tables 2, 3, and 4). The fact that 24.4 percent (84) of the animals had the tumor removed but did not develop metastasis (table 2) indicates that removal *per se* does not provoke metastasis.

Extirpation of the implanted tumor in which there was no recurrence or the recurrence comprised only small latent tumor nodes markedly prolonged the life of the host and thereby greatly increased the duration of the effects of the tumor. However, incomplete excision of all invaded skin or muscle tissue with the tumor usually resulted in early, extensive recurrence and multiple metastasis to axillary and inguinal nodes, often accompanied by invasion of the abdominal cavity followed by death before marked necrosis of the recurrence or cachexia occurred. Few animals having this type of incomplete removal had metastasis to the lungs and none to the kidneys or pancreas Aselli—(mesenteric lymph nodes of Aselli, a group around the superior mesenteric artery).

The methods of implantation employed do not appear to have a definite effect on incidence of metastasis to lymph nodes or lungs (table 1). Subpannicular implantation with brei or blood alone gave 91 to 97 percent takes, but inoculation with solid tumor particles or tumor particles in brei or blood resulted in 100 percent takes.

There was no instance of regression of this tumor. However, if permitted to run its course, the tumor often becomes a necrotic mass of

TABLE 2.—*Incidence of metastasis to lungs, axillary and inguinal lymph nodes, and to both lungs and nodes in 345 hamsters having subpannicular implants for over 15 days*

Duration (days)	Lung metastasis only			Lymph-node metastasis only		
	Hamsters	Removal	Necrotic metastasis	Hamsters	Removal	Necrotic metastasis
	Number			*Number*		
16–23	1	0	0	14	10	2
24–50	14	10	2	45	30	24
51–100+	12	10	7	6	6	4
Total	27	20	9	65	46	30
Percent	7. 8	74. 4	33. 3	18. 9	70. 9	46. 2

Duration (days)	Both lung and lymph-node metastases			No metastasis	
	Hamsters	Removal	Necrotic lung metastasis	Hamsters	Removal
	Number			*Number*	
16–23	0	0	0	69	8
24–50	17	17	8	135	62
51–100+	6	6	5	26	14
Total	23	23	13	230	84
Percent	6. 7	100	56. 5	66. 6	36. 5

TABLE 3.—*Relation of duration and extirpation of the tumor to metastasis*

Duration (days)	Hamsters	Metastasis without removal		Tumor removed		Metastasis to node and/or lungs	
	Number	*Number*	*Percent*	*Number*	*Percent*	*Number*	*Percent*
16–23	84	5	6. 0	18	21. 4	15	17. 9
24–50	211	19	9. 0	57	27. 0	76	36. 0
50–100+	50	2	4. 0	36	72. 0	24	48. 0
Total	345	26		111		115	

TABLE 4.—*Relation of removal and duration of the tumor to number of metastases to axillary and inguinal lymph nodes per animal*

Removal of sarcoma	Hamsters	Duration of tumor in days		Hamsters with node metastasis		Number lymph nodes having metastasis				Total nodes
		Mean	Extremes	Number	Percent	1	2	3	4	
	Number									*Number*
None	173	33. 2	16–64	26	15. 0	22	4	0	0	30
One	142	39. 2	18–83	48	38. 8	19	15	9	5	96
Two	21	55. 4	28–77	12	57. 2	5	4	1	2	24
Total	336			86	25. 6	46	23	10	7	150
LYMPH-NODE REPLACEMENT BY SARCOMA										
Lymphoid tissue remaining in each node						24	5	2	0	40
No lymphoid tissue remaining in any node						22	8	3	1	51
Lymphoid tissue remaining in one of the nodes							10	5	6	59

varying consistency enveloped by a thin subcapsular layer of viable tumor cells.

No evidence of lymphatic or venous extension, or permeation, from implanted or recurrent tumors to lymph nodes was found, even in specially prepared material.

Leukemia, which has been reported for sarcoma-bearing mice (*16*, *18*, *19*), did not occur in any of the tumor-bearing hamsters. However, in a few hamsters neutrophilia produced a mean increase of 47 percent in the polymorphonuclear group.[5]

The occurrence of spontaneous tumor in this colony is rare since autopsy with histologic examination of all structures suspected of being neoplastic of all stock, control and experimental animals to date revealed only one spontaneous neoplasm, a carcinoma.

LYMPH NODES

Axillary and Inguinal

Metastasis to 1 or more of the 6 lymph nodes considered as being regional to the implantation site occurred in 86 (25.6 percent) of the 336 hamsters having subpannicular implants for over 15 days. Metastasis to a single axillary or inguinal lymph node occurred in 46 (53.5 percent) of the 86 animals; to 2 nodes, either 1 or both of the axillaries or inguinals, in 23 (26.7 percent); to 3 of these nodes in 10 (11.62 percent); and to any 4 of the 6 nodes in 7 (8.14 percent) (table 4).

The inguinal nodes were more frequently involved (90, or 60 percent) than the axillary (60, 40 percent), but no significant differences occurred between the nodes of the right and left sides. Forty-six metastases were found in the right inguinal nodes and 44 in the left, while in the axillary nodes 25 secondary growths were found in the right and 35 in the left. The earliest metastases occurred in 4 animals having subpannicular implants for 16 days.

Tumor emboli in lymph nodes were situated chiefly in the cortical areas (Fig. 2, *A*). The developing neoplasm rapidly replaced the entire node (table 4) and often attained a diameter of 35 mm. These large metastases invariably contained liquid necrotic material but did not rupture the capsule. In the 150 involved lymph nodes, the secondary growth had not completely replaced the lymphoid tissue in 49 nodes but had completely replaced it in 99 of the other 101 (table 4). Gross solid or liquid necrotic areas (Fig. 2, *E*) were present in the nodes in 30 of the 65 animals having metastasis only to lymph nodes (table 2).

Replacement of the lymph node (table 4) varied according to the number of foci in it. In a few instances the embolus increased in size as a single solid round mass (Fig. 2, *A* and *B*), but in most cases the secondary tumor grew irregularly and left isolated areas or islands of lymphocytes in various stages of disintegration surrounded by sarcoma (Fig. 2, *D* and *F*). Sections through these islands stained by the Feulgen

[5] Kelsall: Unpublished data.

method showed nuclear fragmentation of the lymphocytes; while those stained with toluidin blue showed concurrent dissolution of the cytoplasm. In hamsters that had not reached the carcinemic stage, sections of noninvolved lymph nodes revealed no increase over controls in number of plasmacytes, such as has been found in the lymph nodes of mice bearing several types of experimentally produced and transplanted sarcomas (*13, 16–19*).

Pancreas Aselli

Metastases to the pancreas Aselli—occurred in 3 of the 345 tumor-bearing hamsters. These metastases were in animals having had removals and a tumor duration of 28, 54, and 61 days. No metastasis to other organs was found in the first of these animals, but in the second there was extensive secondary growth in the axillary and inguinal nodes and lungs; while in the third, metastases were present in these nodes, the lungs, and kidneys.

Lungs

Metastasis to the lungs occurred in 50 (14.5 percent) of the 345 hamsters having implants of more than 15 days' duration (table 2). These secondary growths were multiple and commonly occurred as scattered foci of fairly uniform volume in 3 to all 5 lobes of the lung (Fig. 1, *A* and B). The number of early metastases in the different lobes varied, but the fact that the scattered foci had practically the same volume suggests simultaneous dissemination from the implanted tumor.

Extensive emboli, many of which had penetrated the wall of the vessel, were frequently observed in branches of the pulmonary artery. Penetration of the arterial wall was observed in the areas at which the vessel had been dilated, and a very heavy perivascular infiltration of lymphocytes surrounded these regions of the vessel (Fig. 1, *D* and *E*). The later stages of the secondary growth in the lungs were characterized by progressive fusion of multiple foci with total invasion of one or more lobes and formation of necrotic areas. These necrotic areas were found in 22 of the 50 animals having lung metastases (table 2).

Kidneys, Adrenal Gland, Thymus Gland, and Liver

Metastasis to the kidneys appears to be as rare in the hamster as has been reported for mice (*20*), for this organ was involved in only 3 of the 345 subpannicularly implanted hamsters. Each of these 3 hamsters had one removal and a tumor duration of 41, 48, or 54 days, and each also had metastasis to axillary and inguinal lymph nodes, as well as necrotic, extensive, secondary growth in the lungs.

There was no macroscopic evidence of metastasis in the adrenal gland, thymus gland, or liver of the 345 subpannicular animals, and no embolus or established metastasis was found in sections of both adrenal glands in 95 of these hamsters, in sections of 54 thymus glands, or in sections of 345 livers.

The absence of metastasis to the liver in these 345 hamsters is surprising in view of the occurrence of extensive hepatic involvement in one (duration, 25 days) of 54 hamsters implanted with a benzpyrene-produced, mixed-cell sarcoma (*21*).

The liver was not enlarged as has been reported for rats and mice having sarcomas (*16*, *18*, *28*), but periportal infiltration of lymphocytes and plasmacytes (*14*) occurred in the liver of all the animals having subpannicular implants for 16 or more days.

DISCUSSION

Oberling (*22*) states that transplanted tumors "do not ordinarily produce metastases," although the neoplastic cells spread through the body, and that extirpation of the implanted tumor is "one means of provoking" metastasis. Although he and his coworkers obtained 8 percent metastasis without removal, 56 percent after one, and 82.5 percent after two removals of Jensen's sarcoma in rats, he appears to consider the relation of duration of the tumor to formation of metastasis as being of secondary rather than primary importance. Whether Parson's (*17*) failure to obtain "any trace of metastasis" in 122 transplant generations of Malignant Sarcoma 1 in mice is related to the nature of the sarcoma or to its duration-requirement is not clear. Haddow (*23*) holds that the duration of the primary tumor is a "highly important single factor in determining the occurrence of metastasis" in spontaneous tumors of mice, and Halberstaedter (*21*) made similar observations on hamsters implanted with Sarcoma H. M. The occurrence of metastasis in only 17.9 percent (15) of the 84 hamsters having a tumor duration of 16 to 23 days when compared with 36 percent (76) of the 211 animals having a tumor duration of 24 to 50 days (table 2) shows a significant correlation of metastasis with tumor duration. Although the basis for such conclusions is difficult to quantify, it is evident that in mice (*23*), in rats (*22*), and in hamsters (*21*), as well as in this work, there is a correlation between duration of the effects of the tumor and its metastasis.

That factors other than duration of the tumor affect metastasis is shown by the fact that of the 155 subpannicularly implanted hamsters having 1 or 2 removals and a tumor duration of 24 to over 100 days (table 2), 76 (49 percent) of this group had no metastasis and that of 163 animals having 1 or 2 tumor removals with an average duration of more than 50 days, 44 (26.9 percent) had only 1 metastasis each (20, to the lungs and 24, to 1 lymph node) (table 4).

Development of Metastases

Carefully excised sarcomas showing miliary necrosis or limited invasion of the skin or panniculus carnosus muscle frequently did not recur but before being removed apparently had already invaded the circulation sufficiently to insure metastasis. The number and distribution of the resulting metastases appear to be roughly related to the extent of necrosis

or other lesions within the tumor or in its capsule before or during removal. Thus, in the light of this and earlier work (*1*) it appears that any means by which the thin walls of the vessels within it or the capsule of this sarcoma are opened will provoke recurrence and/or metastasis.

Observations on this tumor in the hamster support the familiar concept that metastases of sarcoma usually reproduce the specific characters of the primary neoplasm (*2*). However, the actively proliferating periphery of primary and of well established secondary growths (Figs. 1, *A*, *B*, *C*, and 2, *B*) and of emboli (Figs. 1, *D*, and 2, *A*) fail to conform to this rule since the neoplastic cells in these situations are all the round type with an occasional atypical spindle type. As the periphery of the metastasis advances, the older areas gradually assume the typical histologic structure of this mixed-cell sarcoma.

Order of Frequency

Many investigators and pathologists hold that in order of frequency of metastasis of sarcomas, the lungs are first and the lymph nodes are second to fifth (*5*, *8*, *25–27*). Moore (*7*) states, "5 to 15 percent of all sarcomas show metastasis in the regional lymph nodes," and Warren and Meyer's (*12*) survey of 237 cases shows that lymph-node metastasis in sarcomas, not including melanoma, lymphosarcoma, and related forms, occurred in 17 (7.2 percent) of the human patients considered. Woglom's (*13*) results, in which he obtained 27 viable node metastases from 14 mice 5 to 27 days after implanting them "subcutaneously" with Sarcoma 37, place the lymph nodes first, although he does not mention metastasis to the lungs. Halberstaedter (*21*) obtained multiple metastases to the lymph nodes in 39 (72 percent) of 54 hamsters implanted with a mixed-cell sarcoma, but only 2 of the animals had secondary growth in the lungs. Because of the heterogeneous nature of the tumors, as well as other variable factors involved, the order of frequency of metastasis to the lungs and lymph nodes of spontaneous sarcomas arising in man and the various laboratory animals cannot be compared with results obtained by transplants of an established line-sarcoma implanted in inbred hosts.

The inguinal and axillary lymph nodes were first and the lungs second as the site of secondary growth in the 115 hamsters having metastasis and a tumor duration of 16 to more than 100 days. Metastasis to lungs only occurred in 27 (23.5 percent) and to lymph nodes only in 65 (56.5 percent) of the 115 animals. Removal of the tumor did not appreciably alter this ratio of 27:65 (1:2.4) since the animals in these two groups that had the tumor removed had ratios of 20:46 (1:2.3), while those that did not have extirpations had ratios of 7:19 (1:2.7). Each of the 23 (20 percent) that had metastasis to both lungs and lymph nodes had the tumor removed (table 2).

SUMMARY

A mixed-cell, or polymorphous, sarcoma, induced by injections of 9,10-dimethyl-1,2-benzanthracene in 1944 and propagated by trans-

plantation, produced 100 percent takes when implanted subpannicularly in inbred Syrian hamsters of various ages and both sexes.

Leukemia was not associated with this sarcoma, and there was no instance of regression of implanted or of secondary tumors.

Inoculation by injecting tumor substance in modified Locke solution in the form of solid fragments, fragments in blood, or in necrotic fluid had no significantly different effect on take, growth, or metastasis of the tumor.

Subpannicular implantation of 345 hamsters having a tumor duration of 16 to over 100 days produced metastasis to the axillary or inguinal lymph nodes or lungs in 115 (33.3 percent) of the animals. Of the 115 hamsters, 65 (56.5 percent) had secondary growths in the axillary or inguinal lymph nodes only, 23 (20.0 percent) had metastasis to both nodes and lungs, and 27 (23.5 percent) had secondary growth in the lungs but not in the nodes. Three of the 115 hamsters had metastasis to the pancreas Aselli and 3 to the kidneys, but none of the 345 tumor-bearing animals had secondary growth, or even emboli, in the liver.

The histologic development of the secondary sarcomas, in which growth of the tumor was effected by round cells, many of which later became fusiform, followed the same course in the lymph nodes, lungs, kidneys, liver, and in various emboli in branches of the renal and pulmonary arteries.

Extirpation of the implanted tumor increased the incidence of metastasis chiefly by prolonging the life of the host until the secondary growths became established. Of the 345 tumor-bearing hamsters, 89 (25.8 percent) developed metastasis following removal of the tumor, while only 26 (7.5 percent) developed metastasis without removal of the tumor.

Incomplete removal of invading tumors resulted in rapidly growing, massive recurrence and multiple, rapidly developing metastases with early death of the host.

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Plate 85

Figure 1.—Metastases to the lungs. *A*, *B*, and *C*, Deep and surface forms. Duration 30 days. Hematoxylin stain. *A*, × 36; *B*, × 65; *C*, × 400. *D*, Embolus in an artery, showing normal and dilated lumen with early rupture and perivascular infiltration of lymphocytes. Duration, 111 days. Toluidin blue stain. × 90. *E*, Detail of the break-through of the embolus shown in *D*. × 300.

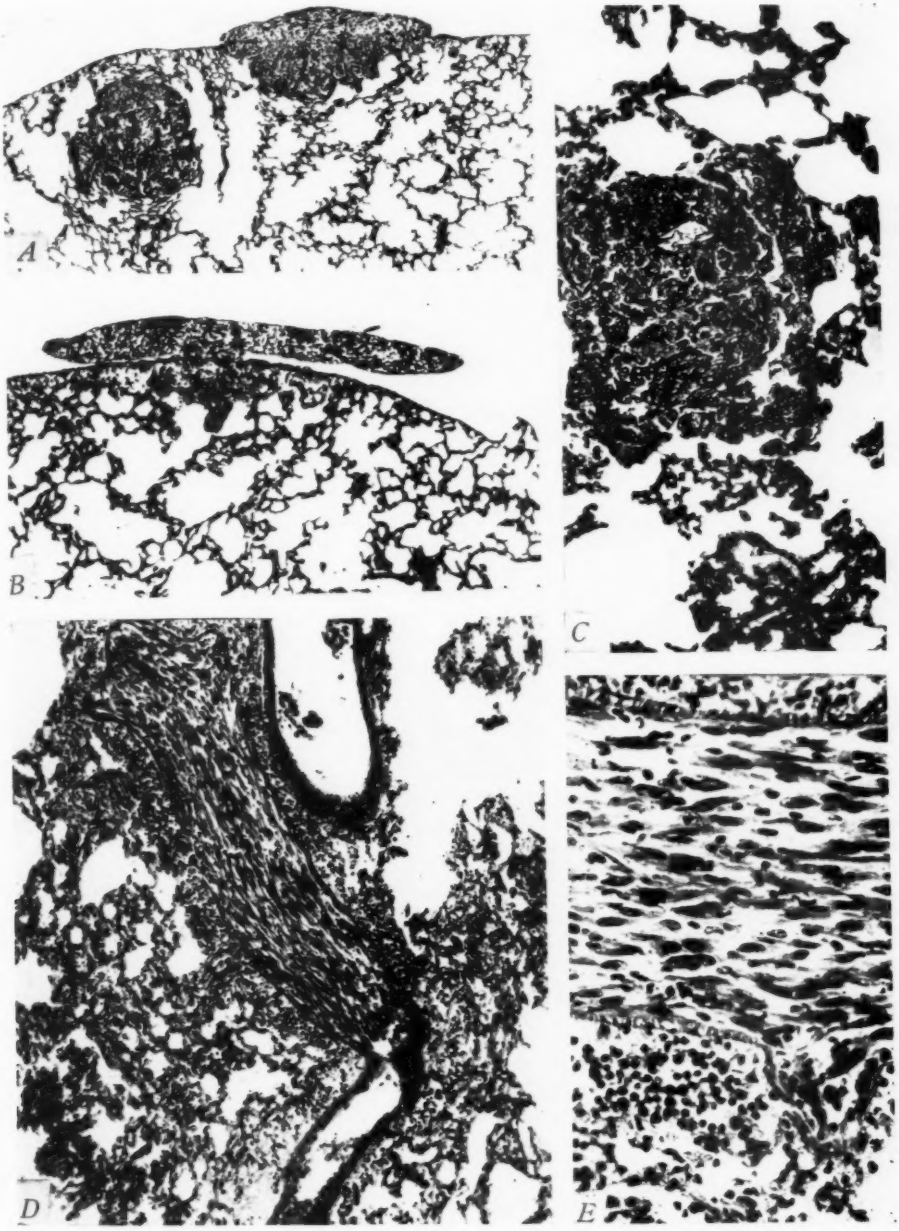
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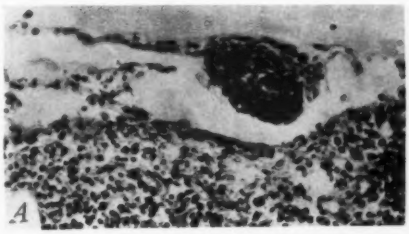
PLATE 86

FIGURE 2.—Metastases to lymph nodes. *A*, Embolus in cortical sinus of pancreas Aselli. Duration 54 days. Toluidin blue stain. × 175. *B*, Replaced germinal center, same node as in *A*. Toluidin blue stain. × 150. *C*, Replacement of left deep axillary node, duration 37 days. Toluidin blue stain. × 150. *D*, Vestige of inguinal lymph node, duration 39 days. Hematoxylin-and-eosin stain. × 350. *E*, Basophilic cytoplasm of tumor cells bordering necrotic center in replaced post-axillary node, duration 64 days. Toluidin blue stain. × 150. *F*, Vestige of pancreas Aselli, duration 32 days. Zenker's base, hematoxylin-and-eosin stain. × 400.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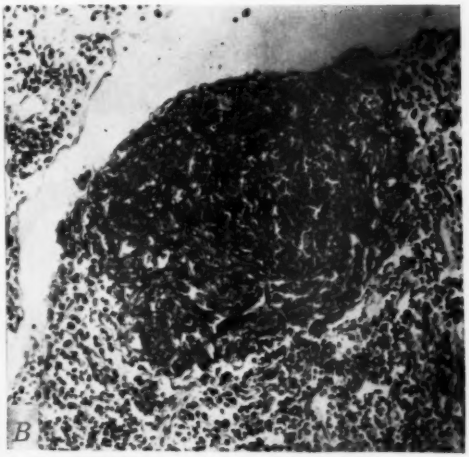
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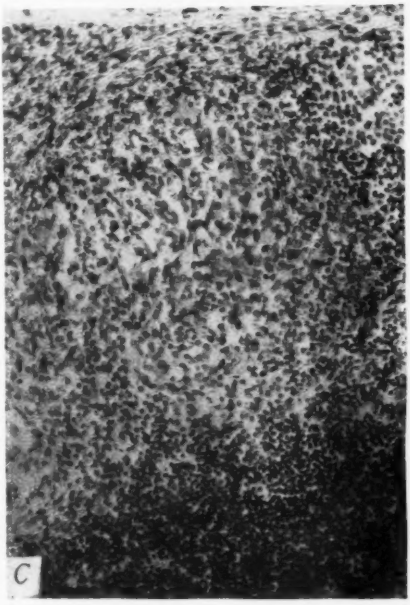
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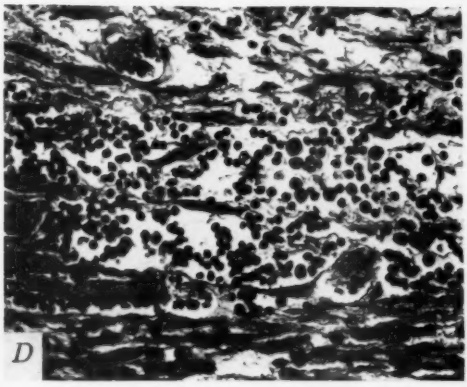
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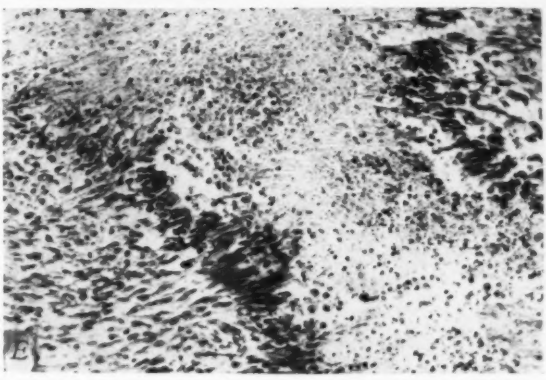
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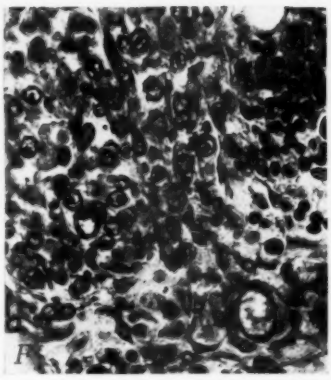
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INDUCED PULMONARY TUMORS IN MICE. IV. ANALYSIS OF DOSE RESPONSE DATA WITH METHYLCHOLANTHRENE [1]

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Previous communications (*1–3*) from the National Cancer Institute have indicated that primary pulmonary tumors are easily and quickly elicited in strain A mice following administration of carcinogenic hydrocarbons. The incidence of tumors and the number of individual tumors per animal are increased as the dose of the carcinogen is increased and as the time following injection is increased (*1*). The same relationship obtains when mice are given repeated doses of ethyl carbamate (*4*). Statistical treatment of such data, however, is not available in the literature. Since the quantitative analysis of dose response data is indispensable in many investigations with biologically active materials, as well as useful in the organization of subsequent studies, an experiment in which strain A mice were given graded doses of methylcholanthrene was initiated in 1944. The present report presents an analysis of the results.

METHODS

The carcinogen employed was 20-methylcholanthrene (m. p. 179.8° C., corrected) dispersed in aqueous cholesterol colloid with the aid of the dioctyl ester of sulfosuccinic acid.[2] The particles of the hydrocarbon in this dispersion were mainly 1.7 μ to 4.0 μ in diameter, with a few long needles. The concentration of the hydrocarbon, 1 mg. per cubic centimeter, was checked spectrophotometrically, and serial dilutions were then made by adding required amounts of the cholesterol colloid.

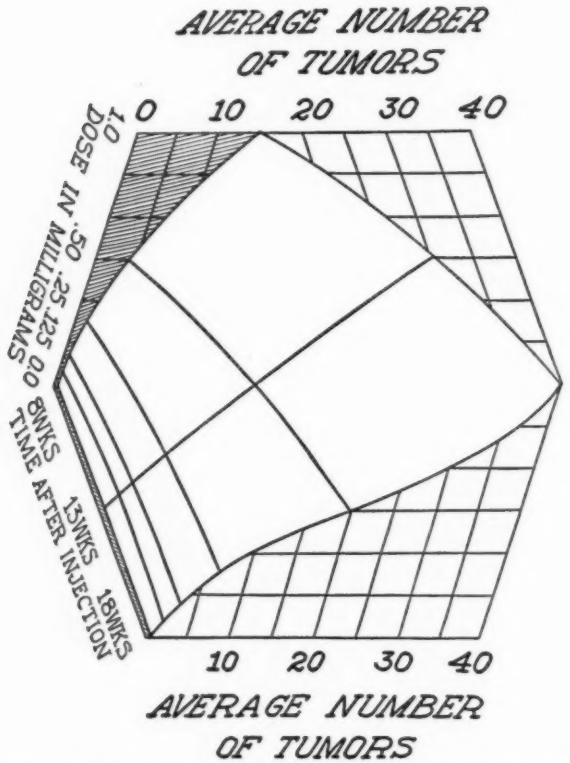
Male mice of strain A 2.5 to 3.5 months old and weighing 18 to 24 gm. were used. They were maintained in wooden boxes, on Purina dog chow and an unlimited supply of water. The animals were divided into approximately equal groups by weight, those weighing 18 to 20, 20 to 22, and 22 to 24 gm. being separated. Sixty mice were kept as untreated controls. Groups of 90 mice received single intravenous injections into a lateral tail vein of 0.0625, 0.125, 0.25, and 0.5 mg. of methylcholanthrene dispersed in 0.5 cc. of the aqueous cholesterol colloid. Another group of 90 mice was given 1.0 mg. of methylcholanthrene in 1.0 cc. in 2 injections of 0.5 cc. about 6 hours apart.

[1] Received for publication July 6, 1949.

[2] Prepared by Dr. Egon Lorenz, by a method described previously (*5*) for the preparation of Dispersion 1.

The mice were killed by cervical dislocation at 8, 13, and 18 weeks following the administration of the carcinogen. Previous data (*1*, *2*) showed that pulmonary tumors are produced within 4 weeks following intravenous injection of methylcholanthrene but that with larger doses the tumors tend to coalesce and become difficult to count at 6 months following the procedure.

The lungs were fixed in Tellyesniczky's fluid and examined for the presence and number of the typical white nodules on the surface. The great majority of the tumors, particularly when only a few are present, are subpleural and easily visible on the surface of the lung. With large numbers of tumors, deeper nodules are present and not included in the enumeration that would be complete only by serial section of each lung, a procedure precluded by practicability. Since the gross appearance of primary pulmonary tumors in mice is typical and the histological appearance has been described fully elsewhere, only a few representative sections were taken for histologic verification.



TEXT-FIGURE 1.—Relationship of number of tumors to dose of methylcholanthrene and time after injection.

RESULTS

The numbers of tumors developing in 8, 13, and 18 weeks with various dosages are shown in table 1. Text-figure 1 indicates the increase in the number of tumors both with increasing time and with increasing dosages of methylcholanthrene.

TABLE 1.—*Distribution of number of tumors in relation to size of dose and time after injection*

Dose	Time after injection	Mice with pulmonary tumors, according to number of tumors per mouse															Total mice
		0	1	2-5	6-10	11-15	16-20	21-25	26-30	31-35	36-40	41-45	46-50	51-55	56-60	Over 60	
Milligrams	*Weeks*																*Number*
1.0	18				2	2			1	2	6	4	4	1		3	25
1.0	13			4	1	2		3	4	5	2	1	2	4		1	29
1.0	8			2	9	6	5		1	4							27
0.5	18		1	3	5	1	6	4	1	2	4						27
0.5	13	2	1	3	7	7	4	2		2		1					29
0.5	8		6	14	5	3											28
0.25	18	1	1	13	12	2											29
0.25	13		1	13	12	1											27
0.25	8	6	7	16	1												30
0.125	18	3	7	16	2												28
0.125	13	5	6	17	2												30
0.125	8	18	6	5													29
0.0625	18	11	8	11													30
0.0625	13	11	9	9	1												30
0.0625	8	15	10	4													29
0.000	18	24	4	2													30
0.000	8	27	3														30

Means and standard deviations of the means for each time interval and each dose are given in table 2. With increased dosages and increased time, the mean number of tumors and the standard deviation both increase. A small, although not significant, increase in the number of tumors with time also occurred in the untreated controls. There was no significant difference in the number of tumors elicited in mice of different body weight groups.

Significance of the differences between mean numbers of tumors found in hosts receiving various amounts of methylcholanthrene but all kept the same time after injection was determined by means of Student's t (*5*) in which

$$t=\bar{x}\sqrt{\frac{n_1 n_2 (n_1+n_2-2)}{(n_1+n_2) S x^2}}. \qquad [1]$$

In all instances the differences were significant at the 1 percent level except when the difference in the mean number of tumors resulting from the injections of 0.125 and 0.0625 mg. with a waiting time of 8 weeks was considered. In this instance the mean number of tumors was greater for 0.0625 mg. than for 0.125 mg., but the difference was not significant.

It may be concluded that, in general, for the number of mice used per sample and for the time intervals employed, the dosages differed in amount sufficiently to produce significantly different numbers of tumors in the hosts.

TABLE 2.—*Mean numbers of tumors and standard deviations for various doses and times after injection*

Dose	Time	Mice	Average tumors	Standard deviation
Milligrams	*Weeks*	*Number*	*Number*	
1.0	18	25	40. 48	17. 98
1.0	13	29	30. 31	16. 78
1.0	8	27	14. 96	9. 25
0.5	18	27	19. 30	11. 83
0.5	13	29	13. 55	10. 08
0.5	8	28	4. 39	3. 46
0.25	18	29	5. 55	2. 88
0.25	13	27	5. 37	2. 86
0.25	8	30	2. 03	1. 58
0.125	18	28	2. 61	1. 87
0.125	13	30	2. 40	1. 76
0.125	8	29	. 62	. 96
0.0625	18	30	1. 27	1. 66
0.0625	13	30	1. 23	1. 38
0.0625	8	29	. 66	. 80
Control	18	30	. 30	. 52
Control	8	30	. 10	. 31

Significances of the differences between mean numbers of tumors in mice kept different lengths of time but receiving the same amount of methylcholanthrene were also determined (table 3). Differences were significant at less than the 1 percent level in all cases where comparison was made between mice kept 8 weeks and those kept 13 and 18 weeks and where injections were of 0.125 mg. or more.

TABLE 3.—*Level of significance of differences between groups with dose held constant*

Dose	Time in weeks		
	18 vs. 13	18 vs. 8	13 vs. 8
Milligrams	*Percent*	*Percent*	*Percent*
1.0	4	1	1
0.5	6	1	1
0.25	82	1	1
0.125	66	1	1
0.0625	94	4	6
Control	----------	14	----------

DISCUSSION

The experiment demonstrates that the lungs of strain A mice are a sufficiently sensitive test medium for methylcholanthrene, and probably other carcinogens, to distinguish doses differing from one another by a factor of two. Statistically significant differences are obtained when

approximately 30 mice are used per group. This sensitivity applies within certain limits, and when the intravenous route of administration is utilized.

The number of pulmonary tumors elicited by intravenous administration of carcinogenic hydrocarbons is dependent upon the dose retained by the lungs, which is dependent upon the size of the particles in the dispersion (*3*). The dispersion used consisted of particles 1.7 to 4.0 μ in diameter and averaging approximately 2.5 by 2.5 μ. It was determined (*3*) that upon intravenous injection of 0.1 mg. of this dispersion approximately 10 percent became lodged in the lungs. The wide range in the number of tumors was not attributable to an uneven distribution of the particulate carcinogen, since comparison of these data with those of Henshaw and Miller (*4*), who elicited pulmonary tumors by the intraperitoneal injection of urethane, show that the standard deviations are not significantly different.

In connection with many experiments the question arises about the optimum number of mice to use per experimental group in a repetition of the experiment. The question is capable of solution with varying degrees of exactness depending upon what assumptions are made. For example, these assumptions might be that (1) the population being considered is normally distributed with respect to the trait in question, (2) in each of a pair of groups being compared the numbers of mice are the same or nearly the same, (3) no new factors are to be introduced into the experiment in a subsequent phase of the study, (4) the ratio $\frac{s_1^2+s_2^2}{\bar{x}^2}$ [a] will not become larger as the size of the sample is changed, and (5) the level of significance at which differences between means of groups will be considered as not ascribable to chance is established in advance.

From equation (1)

$$t^2=\bar{x}^2\left[\frac{n_1n_2(n_1+n_2-2)}{(n_1+n_2)Sx^2}\right]$$

letting $n_1=n_2=n$, this becomes

$$t^2=\bar{x}^2\left[\frac{n(n-1)}{Sx^2}\right]. \qquad [2]$$

Now $Sx^2=\Sigma x_1^2+\Sigma x_2^2$ and

$\Sigma x^2=s^2(n-1)$ by definition.

Hence, substituting in equation (2), we have

$$t^2=\bar{x}^2\frac{n}{s_1^2+S_2^2},$$

Or, solving for n,

$$n=\frac{t^2}{\bar{x}^2}(s_1^2+s_2^2).$$

---

[a] $\bar{x}$ is the difference of the means of the two samples compared. s_1^2 and s_2^2 are the variances of the two samples compared.

When the formula is applied to the data of table 3, the estimated number of mice needed to reach the 1 percent level of significance can be calculated (table 4). The indicated irregular increase in the number of mice needed as the dose is decreased is due to the inadequacy of the sampling when the doses were small in the present experiment. A more accurate prediction of the number of mice required might be obtained by taking successive averages by threes of the adjacent numbers shown in table 4.

For the preparation of table 4, t was taken as 2.58, the smallest value of t which indicates significance at the 1 percent level regardless of the size of the sample. With decreasing values of n, the 2.58 value of t is considerably in error, and the numbers shown are, therefore, too small. Furthermore, although the data of table 4 were based upon the assumption that the difference between the means would not become smaller or at least that the ratio $\frac{s_1^2+s_2^2}{\bar{x}^2}$ would not become larger when the new samples were drawn, this condition is likely to be realized in practice no more than 50 percent of the time.

TABLE 4.—*Estimated number of mice needed in order to achieve significant differences*

Dose	Number of mice		
	18 *vs.* 13 weeks	18 *vs.* 8 weeks	13 *vs.* 8 weeks
Milligrams			
1.0	35	5	11
0.5	49	5	9
0.25	3, 400	6	7
0.125	990	10	9
0.0625	15, 000	41	52
Control	----------	60	----------

If the experimenter is interested in assuring himself that his differences, whatever they may turn out to be, are significant, more refined techniques are available (*6*) for estimating the number of mice needed to account for the possibility that differences between means obtained in a replication of the experiment may be smaller than those obtained originally. For example, if the experimenter wished to be 95 percent certain that differences obtained in the replication of the experiment will be significant at the 1 percent level, 2.7 times as many mice would be needed in each group. For 99 percent certainty, 3.6 times as many mice would be needed as is indicated in table 4.

In addition to these considerations, it is likely that the variances s_1^2 and s_2^2 will not be the same when the experiment is repeated and that the procedures employed in conducting the experiment will also differ slightly. Hence, the actual decision as to the number of mice to use must be made partly on the basis of the statistics and partly according to the subjective judgment of the experimenter.

SUMMARY

A statistical analysis is presented of data on the induction of pulmonary tumors in strain A mice following single intravenous doses of 0.0625 to 1.0 mg. methylcholanthrene.

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REMOTE-CONTROLLED RADIUM EMANATION PLANT AND AUTOMATIC MEASURING APPARATUS FOR GOLD IMPLANTS [1] [2] [3]

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RADIUM EMANATION PLANT

The radium emanation plant installed in the U. S. Marine Hospital is a variation of the original Failla design [5] developed by G. Failla and the senior author in 1939. It is equipped for semi-automatic operation and is unique in that remote control of the stopcocks has been incorporated into the installation. This lends itself admirably to reduction of exposure of personnel to radiation. The electrical control apparatus for semi-automatic operation and remote controls for the operation of stopcocks are located in a control room (Fig. 1) separated from the emanation plant by 2 feet of concrete. A lead glass window and a low-power telescope are mounted in the wall for the observation of the operation of the plant.

A view of the emanation plant as seen from the control room through the lead glass is shown in Figure 2. Since the apparatus consists of glassware which must be replaced occasionally, because of embrittlement, and since some of the parts, such as the copper oxide tube and the phosphorus pentoxide tube, must be replaced owing to use, two complete systems have been built, one mounted on each side of a V-shaped frame. It is so arranged that either side may be used in order that there will be no delay in the collection of radon if one of the sides is not in operating condition.

Since the two systems are duplicates, only one side will be discussed. A diagrammatic representation of the system is shown in text-figure 1. Stopcock D is operated by the upper handle in the control room, and stopcocks A, B, and C are operated by the lower handle.

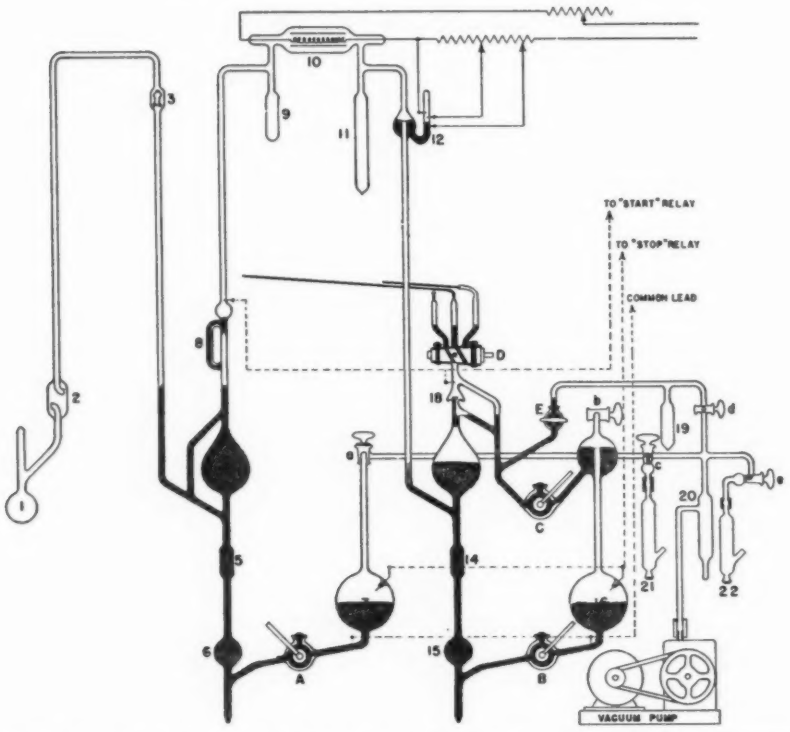
[1] Received for publication August 5, 1949.

[2] A description of these units was presented at the American Radium Society by J. E. Rose at the June 1942 meeting and by R. W. Swain at the June 1949 meeting.

[3] The authors wish to acknowledge the assistance of the Leeds and Northrup Co. in the design of the measuring and sorting apparatus and for the use of the schematic and wiring diagrams for the preparation of text-figures 6 and 7. They also wish to acknowledge the excellent machine work by Mr. J. W. Sweat and the cooperation of Mr. W. Maginnis in the preparation of the illustrations.

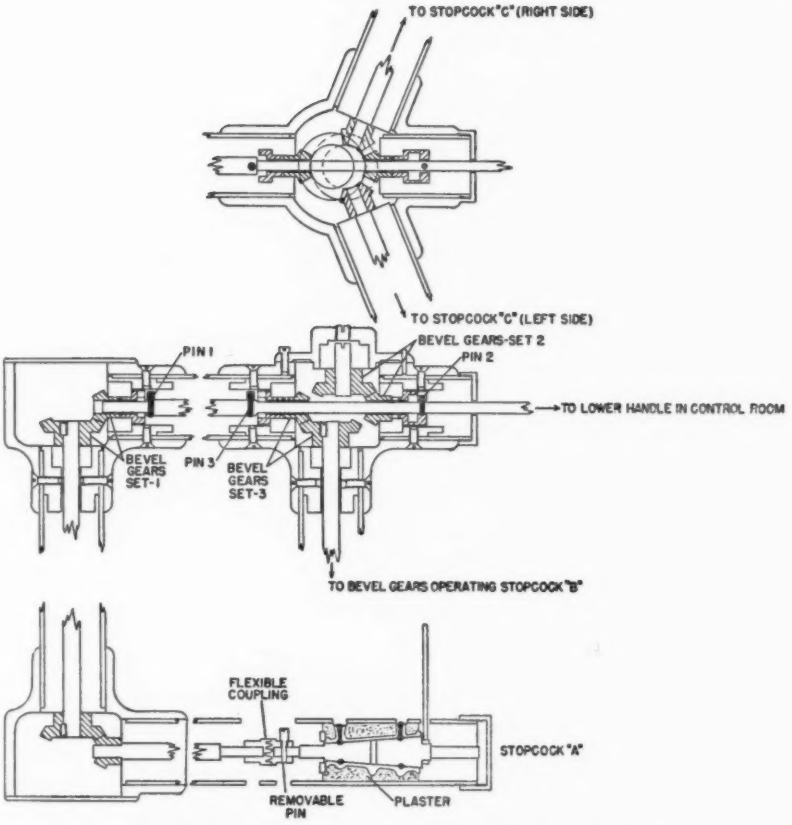
[4] Now at Argonne National Laboratory, Chicago.

[5] G. Failla, U. S. Patents No. 1,553,794 and No. 1,609,614.



TEXT-FIGURE 1.—Diagram of remote-controlled semi-automatic radium emanation plant.

Text-figure 2 shows the details of the remote control for stopcocks A, B, and C. The position of the handle toward or away from the wall determines the stopcock to be operated. A pin on the shaft as it goes through the wall engages in slots in a stationary bushing to assure correct positioning of the shaft to operate only one stopcock and to restrict the shifting of operation from one stopcock to another only when they are all in the closed positions. The shaft through the wall is connected to the main drive shaft by a universal coupling. Moving the handle to its position nearest the wall engages pin 1 to operate bevel gears Set 1, and turning the handle operates stopcock A. In the central position as shown in the figure, pin 2 is engaged to operate bevel gears Set 2 to operate stopcock C. In the outer position, pin 3 is engaged to operate bevel gears Set 3 to operate stopcock B. The ratios of the bevel gears are such that 90-degree rotation of the handle opens and closes the stopcocks. To change the operation from one side to the other, the removable pins are removed from one side and replaced on the other side.



TEXT-FIGURE 2.—Details of remote-control mechanism.

Because of the strain produced by the method of applying torque to the stopcock plugs, these are made from stainless steel. All the stopcocks through which radon passes are mercury-sealed and graphite lubricated.

The radium source for the radon is a weak hydrochloric acid solution stored in a lead-lined safe. The glass tubing coming through the top of the safe to a little below the surface of the mercury in the tube leading from trap 3 to reservoir bulb 4 is shielded with lead. The safe is cooled by water flowing through the copper tubing at the back of the safe to promote condensation in this line. A heater coil is placed around the tubing leading from trap 3 to reservoir bulb 4 to reduce condensation. A housing is placed around reservoir bulb 4, and a 100-watt lamp is placed in the housing as a source of heat to reduce condensation at this point. Excessive evaporation of the solution would require frequent replenishment and consequent exposure of personnel to radiation.

In the cabinet below the safe are located the vacuum pump and gas and air lines for glass blowing.

The complete system (see text-fig. 1) except for space above the mercury in reservoirs 7, 16, and 17 is maintained under a vacuum of approximately 0.2 mm. of mercury at all times. The levels of mercury shown in the figure are in the standby position.

Collection of Radium Emanation

The radon is emanated by the radium chloride solution in flask 1 and is collected in the tubing between the flask and the surface of the mercury in the tube leading from trap 3 to reservoir bulb 4. Trap 2 prevents solution from getting into the collecting line, and trap 3 prevents mercury from getting into the solution if a break should occur in the purification section. Included with the radon are impurities consisting of water vapor, hydrogen, and oxygen formed from dissociation of the water, chlorine, and helium which is formed by recombination of the alpha particles. Because of the vapor pressure of this mixture, the pressure in the line below trap 3 is greater than that between reservoir bulb 4 and the loop trap 8. In order to reduce the danger of breaking trap 8, it is the usual practice to pump manually until these pressures are equalized. The vacuum pump is started and stopcocks *a* and *c* are positioned to place a vacuum on reservoir 7. Stopcock A is opened, allowing the mercury in bulb 4 to fall. The radon bubbles through the side arm to the point where the mercury in the loop trap is fully depressed. This volume of gas is then pushed through the trap by turning stopcock *c* so that atmospheric pressure on the mercury in reservoir 7 pushes the mercury up with the gas ahead of it. This procedure is repeated until the pressure in the line below trap 3 is about equal to the pressure above trap 8, then it is set for automatic operation. Stopcock *c* is opened to the vacuum pump, and stopcock *e*, which is located in the remote control unit, is set to leak at a rate which is not sufficient to reduce the vacuum to a point where the mercury does not fall but is sufficient to give a cycling rate of about one stroke per minute. Stopcock A is opened, and as the mercury falls with the vacuum pump on, the mercury level in reservoir 7 rises until it reaches a contact. The contact operates a relay which stops the vacuum pump. Air passing through stopcock *e* then pushes the mercury up, forcing the gas in reservoir bulb 4 ahead of it through trap 8 until the mercury reaches the contact above trap 8. This contact operates a relay which starts the vacuum pump. Allowing this cycle to repeat itself 8 to 10 times is sufficient to place over 95 percent of the radon which has formed since the previous pumping in the system above trap 8.

Above trap 8, the radon and its impurities diffuse through the NaOH tube 9 which removes the chlorine over a platinum heater which acts as a catalyst in the recombination of the hydrogen and oxygen and through heated copper oxide cylinder 10 which removes the excess hydrogen, through the P_2O_5 tube 11 which removes the recombined hydrogen and

oxygen and the water vapor, to the surface of the mercury in the tube below the manometer 12.

As the pressure in this section rises, the mercury in the manometer rises, due to the influx of the emanation, shunting some of the resistance in series with the heating element. This compensates for the drop in temperature due to the presence of gas molecules, thus increasing the rate of purification. As the pressure is reduced due to the removal of the impurities, resistance is added so that the life of the heating element is maintained at a maximum. The purification is allowed to take place until the pressure has dropped to a minimum as indicated by the glow of the heating element and also by measurement of the current through the heating element. The time required is in the order of 10 minutes.

The radon is then ready to be put into its container, which may be glass capillary tubing, a glass bulb, or gold capillary tubing, or it may be subdivided among any combination of the three, depending upon its intended use. If it is to be used in place of radium in applicators, it is sealed into the glass capillary tubing. If it is to be used for its beta ray activity, it is sealed into a thin-walled glass bulb. If it is to be used interstitially, it is sealed into gold capillary tubing.

Stopcock D is turned so that it is opened to the glass capillary tubing or to the glass bulb if all the radon is to be put into the bulb. The residual gases are measured by opening stopcock e to place atmospheric pressure on mercury in reservoirs 16 and 17. Opening stopcock B will then force the mercury in bulb 13 to atmospheric height above the level of the mercury in reservoir 16. Opening stopcock C will force the mercury to atmospheric height above the level of the mercury in reservoir 17. The amount of residual gas is measured by the length of capillary tubing not filled with mercury. A vacuum is then placed on reservoirs 16 and 17, and the mercury is lowered first into reservoir 17 so that the mercury is level with the overflow tube into reservoir 16. The platinum ball trap 18 is constructed so that if the mercury passes through it rapidly, the inertia of the mercury will cause it to close, and a pool of mercury left above it will keep it closed. If the mercury passes through it slowly, the platinum ball will float to the top of the mercury and remain open allowing all the mercury to drain through.

Stopcock B is then opened, and the mercury falls out of reservoir 13 until it is connected to the purified radon which diffuses into the reservoir. When the mercury in reservoir 16 reaches the contact, the vacuum pump stop relay is energized stopping the vacuum pump and air leaking through 22 and e pushes the mercury up and forces the gas in bulb 13 through trap 18. When the mercury reaches the contact above the trap, the start relay is energized, and the vacuum pump is started. This is continued for 15 to 20 cycles until by measurement the increase in volume per stroke is negligible.

The radon may then be divided and placed into the gold capillary tubing and/or the glass bulb using the volume of the radon in the glass

capillary tubing as a measuring device. The glass bulb or the glass tubing containing the gas is sealed off with a gas flame. The gold capillary tubing is cut off with a pair of diagonal cutting pliers.

The glass capillary tubing is cut into lengths of 1.5 cm. and sealed in color-coded platinum sleeves with paraffin. The sleeves are 1 mm. inside diameter with a 0.5 mm. wall which serves to filter out most of the undesired beta radiation.

The gold capillary tubing is cut into lengths of 3.5 mm. It is 24 carat gold which is self-sealing when cut, due to its malleability. It has a 0.15 mm. bore and a 0.3 mm. wall.

AUTOMATIC MEASURING APPARATUS FOR GOLD IMPLANTS

The radon implant measuring apparatus was designed and built in 1941 under the direction of one of the authors (J. E. R.) in the interest of more accurate clinical evaluation of the results of radiation from radon implants [6] and with a view toward the reduction of exposure of personnel to radiation.

In some laboratories, the implants obtained from a single collection of radon are lumped together and measured *en masse*, the total activity being divided by the number of implants to obtain the activity of each implant. This is not desirable since the implants are not uniform in activity, and it is possible that some implants may have no activity and the remainder a greater activity due to flaws in the capillary tubing or variations in length of the implants.

In other laboratories, each implant is measured individually, which is generally a laborious undertaking and results in considerable exposure of personnel if a large number of implants are required.

This apparatus automatically measures and records the activity of each implant and deposits it into a compartment corresponding to its range of activity. It consists of four separate units: (1) The Failla radioactivity recorder, (2) the electrometer tube unit, (3) the auxiliary relay panel, and (4) the measuring cabinet.

Failla Radioactivity Recorder

The Failla radioactivity recorder (Fig. 3) is the control unit for the apparatus. In it are contained the following:

(1) The program switch which determines the durations and sequences of operations during the measuring cycle;

(2) The low-voltage power supply which supplies direct current for the electrometer tube circuit;

(3) The high-voltage power supplies, one of which supplies direct current for the recorder slidewire, the other of which supplies direct current for the ionization chamber and operation of relays;

[6] Throughout this paper, due to common usage, the terms implants, seeds, and gold tubes are used interchangeably.

(4) The current standardizing unit which standardizes the current in the electrometer tube circuit;

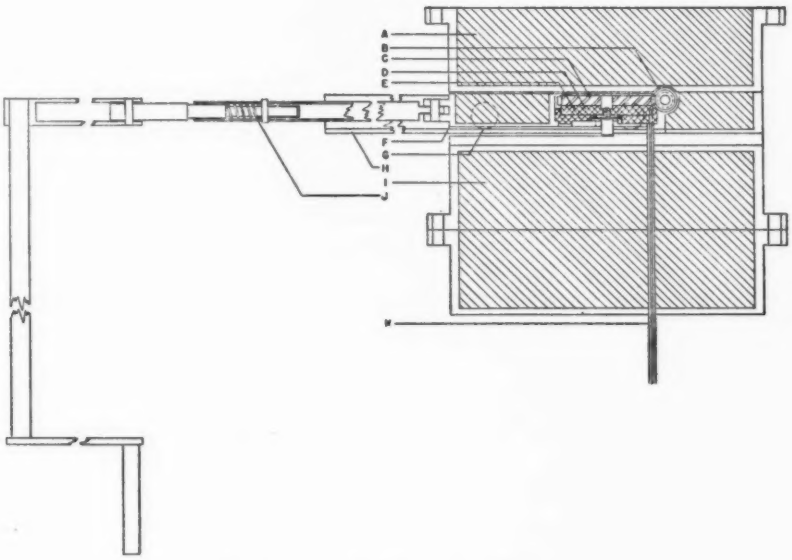
(5) The voltage standardizing unit which standardizes the recorder slidewire voltage;

(6) The recorder unit which is a variation of the Leeds and Northrup Micromax Recorder. This is a device which transforms the deflection of a galvanometer into rotation of a shaft. The rotation of the shaft is transmitted mechanically to the recording pen and pointer. At the same time the slidewire on the shaft is rotated so that the voltage on the slider is varied. Also on the shaft of the recorder is located the contact arm of the segmented selector switch which is used to select the pocket in the receiver disc into which the implant is placed.

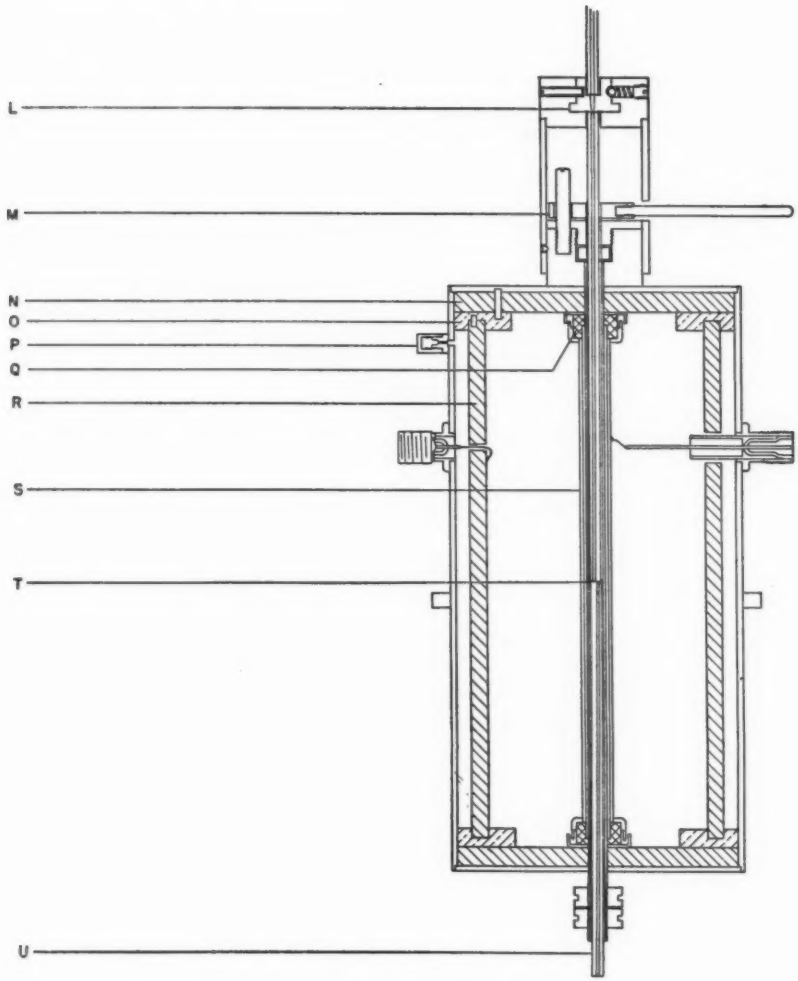
(7) Electrometer tube compensating network which is in effect a type of bridge circuit in which various resistors may be adjusted so that reasonably small changes in current from the power supply to the network will not disturb the galvanometer. When the circuit is properly adjusted, the galvanometer responds only to changes in grid potential.

Measuring Cabinet

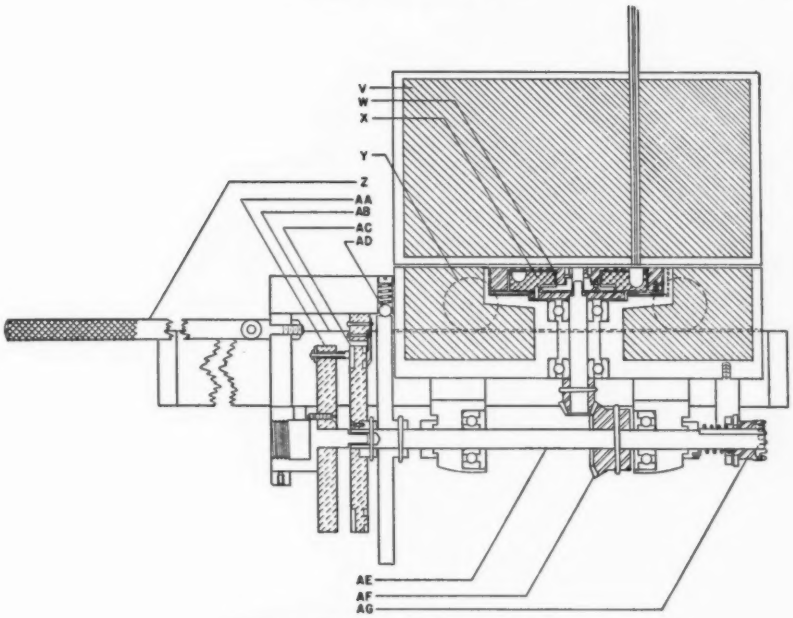
The measuring cabinet consists of three main sections: (1) The upper magazine (text-fig. 3), (2) the ionization chamber (text-fig. 4), and (3) the lower receiver (text-fig. 5). The implants drop by gravity from the upper magazine through a brass tube into the ionization chamber where they are measured, and upon ejection from the ionization chamber, they drop by gravity into the lower receiver through another brass tube.



Text-figure 3.—The upper magazine.



TEXT-FIGURE 4.—The ionization chamber.



TEXT-FIGURE 5.—The lower receiver.

UPPER MAGAZINE

The brass disc B into which the implants were placed by the cutting machine is placed inverted in the carrier in the upper magazine. The carrier rides on ball bearings G on a brass track H. By means of a lever action and transom type locks, the carrier F is moved into place between two lead blocks, A and I, the teeth of the worm gear C being meshed with a worm B mounted on a drive shaft. A flat key projecting from the cover E of the brass disc sliding into a guide assures the alignment of the exit hole of the cover with the delivery tube to the ionization chamber.

At one end of the drive shaft on which the worm is mounted is a sprocket which is operated by chain drive from a motor mounted below. On the other end of the shaft there is a contact disc on which are mounted brass segments. A stationary spring-loaded contact acts as a brush. The circuit is arranged so that if the motor is energized by relay CR2 for a sufficient length of time so that the large segment reaches the brush, it will continue to be energized, rotating in the direction of the arrow until the space between the segments is reached. If the inertia of this system is so great that this space is overshot, the motor will reverse by virtue of the smaller segment being connected to the reverse winding of the motor until the shaft is aligned so that this dead spot on contact disc is reached. The ratio of the worm and worm gear is 1 to 50 so that one revolution of the shaft rotates the disc from one implant containing hole in the disc to the next.

This process allows one implant to fall through the hole in the cover of the disc at the beginning of each cycle in the program. The implant drops into the delivery tube to the ionization chamber.

Ionization Chamber

The ionization chamber is of the concentric cylinder type with a hole axially through the center. The outer electrode R is ⅛-inch thick lead serving as a shield from external radiation as well as an electrode. The two ends of the chamber are also shielded from external radiation by means of two ½-inch thick lead discs N. The outer electrode is insulated from the end shields by glass discs O. The inner electrode is a brass tube S which is supported by and protected from the end shields by quartz insulators Q.

The ionization chamber is in an air-tight brass container consisting of two end discs soldered to a brass cylinder with a brass tube passing through the center.

The voltage leads for the ionizing potential are introduced into the chamber by means of a fernico tube, pyrex insulator, and fernico sleeve. The lead for the central electrode is provided with a grounded shield to minimize field distortion.

The tube through which the implant travels passes through the central tube of the ionization chamber. It is split into two sections at T, and the holes in each section are eccentric so that the holes do not line up unless one is oriented properly with respect to the other by rotation. Thus the implant drops through the delivery tube from the upper magazine until it strikes the section of the delivery tube to the receiver magazine. The implant remains in this position until a late phase of the program cycle at which time the upper tube is rotated by a solenoid-operated arm M which is attached to the tube at the upper end of the ionization chamber.

The upper end of the ionization chamber is provided with a slide arrangement L, so that the delivery tube from the upper magazine may be moved aside and replaced by a small funnel for the measurement of a standard radium cell or for the measurement of individual seeds.

A tube P on the side of the ionization chamber has been provided for evacuating the chamber and filling it with dry air at atmospheric pressure.

Lower Receiver

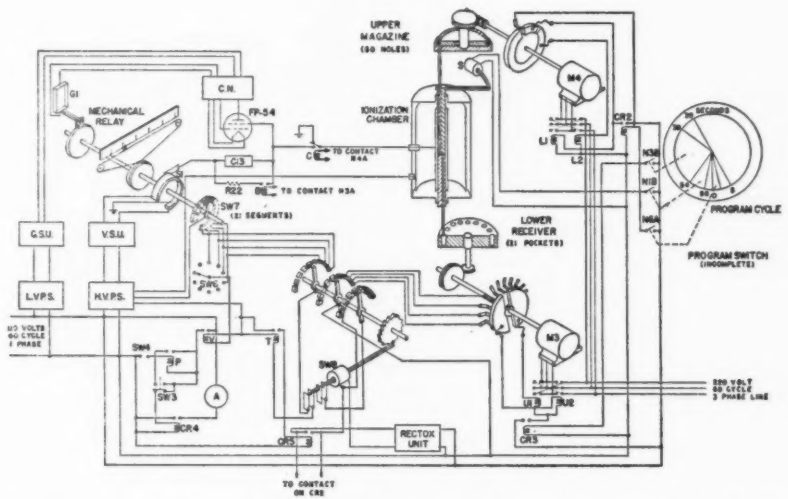
The lower receiver disc is constructed on somewhat the same general principle as the brass disc in the upper magazine, the disc X with the pockets being rotated inside a cover W by means of a pin engaging in a hole in the bottom. The pockets in this disc are much larger since each will contain more than one implant. The cover is also keyed so that its hole will line up with the delivery tube from the ionization chamber.

There are 21 pockets in this disc, the first of which receives all implants of zero activity (duds), the second receives implants ranging from 0 to 0.5 millicuries, and the remaining pockets receive implants with logarithmically increasing activity.

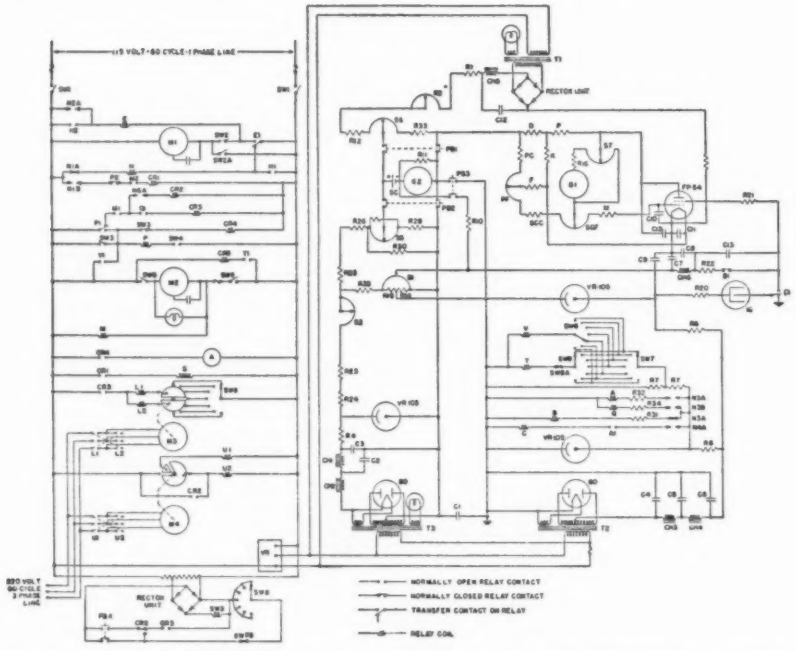
The entire mechanism except for the driving motor is mounted on the carrier for the receiver disc. On the drive shaft AE at one end is half of a multijawed coupling AG which engages the other half mounted on the motor, when the carrier is in position between the lead blocks. A bevel gear AF is mounted on the shaft to rotate the receiver disc. On the other end of the shaft is a contact disc AC on which are located two brass segments. These segments are connected to the motor through slip rings on the opposite side of the disc in such a way that power applied to one segment will cause the motor to rotate clockwise and when applied to the other segment will cause the motor to rotate counterclockwise. There are 21 brushes mounted on a stationary lucite disc M which ride on the segments of the lucite disc attached to the drive shaft. Each of these brushes corresponds to a range of activity and to a pocket in the receiver disc. At the end of the measuring phase of the program cycle, the brush corresponding to the activity of the implant being measured receives power, and the motor rotates the disc until the lucite disc has positioned itself at the point where the brush is between the segments. The implant is then ejected from the ionization chamber and falls into this pocket in the receiver disc.

OPERATION

The operation of the measuring apparatus is best described by reference to the schematic diagram in text-figure 6. Identification symbols on this diagram correspond to those on the wiring diagram, text-figure 7.



TEXT-FIGURE 6.—Schematic diagram of automatic measuring apparatus. C. N., compensating network; C. S. U., current standardizing unit; V. S. U., voltage standardizing unit; L. V. P. S., low-voltage power supply; H. V. P. S., high-voltage power supply.



TEXT-FIGURE 7.—Wiring diagram for automatic measuring apparatus. Contacts N1A to N6A are contacts on the program switch.

All operation is controlled by the program switch which is driven by a 1-r. p. m. synchronous motor. Cams on the shaft of the motor operate contacts which produce the following operations in sequence. Stated times represent elapsed time after program switch motor has started:

(1) Contact N6A is closed from ½ second to 2½ seconds. This energizes relay CR2 which resets the recorder to zero and places voltage on the small segment of the contact ring controlling the upper magazine. This in turn operates the motor through one revolution and aligns one of the holes in the disc containing the gold implants with the hole in the cover, and an implant drops by gravity into the ionization chamber.

(2) Contact N4A is closed from 5 seconds to 40 seconds. Relay C is energized, switching the ionization chamber from ground to the grid of the FP–54 electrometer tube, and the measurement begins. The recorder galvanometer operates the mechanical relay turning the recorder shaft. On the shaft is mounted the drive mechanism for the indicator which travels in front of the recorder scale, the recorder slidewire which, when the voltage is balanced against the FP–54 grid voltage, terminates the travel of the recorder, and a segmented switch which is graduated logarithmically. The segmented switch contains 21 segments which are energized as shown in table 1. The segmented switch performs two functions: (a) To operate the stepping relay one step per segment, and (b) to operate the alarm through relay V and relay CR4 when a preselected segment has been reached.

As the stepping switch is operated by the segmented switch through the back row of contacts, the second row of contacts places voltage on the brushes for the lower magazine pocket selector.

TABLE 1.—*Energized segments*

Segment	Range	Mean value	Segment	Range	Mean value
1	0. 00–0. 01	0. 005	12	1. 49–1. 67	1. 58
2	. 02– . 50	. 26	13	1. 68–1. 89	1. 80
3	. 51– . 55	. 53	14	1. 90–2. 13	2. 02
4	. 56– . 63	. 60	15	2. 14–2. 44	2. 28
5	. 64– . 71	. 68	16	2. 45–2. 72	2. 59
6	. 72– . 80	. 77	17	2. 73–3. 07	2. 90
7	. 81– . 91	. 87	18	3. 08–3. 47	3. 28
8	. 92–1. 03	. 98	19	3. 48–3. 91	3. 70
9	1. 04–1. 16	1. 11	20	3. 92–4. 42	4. 18
10	1. 17–1. 31	1. 25	21	4. 43–5. 00	4. 70
11	1. 32–1. 48	1. 40			

(3) At 35 seconds, contact N5A closes operating relay A. Contact A1 opens and deenergizes relay C. The ionization chamber lead is grounded, and the measurement is terminated.

(4) From 39 seconds to 58 seconds contact N3A is open, deenergizing relay B. The charge on the air condenser is by-passed through contact B1 and resistor R20, and the recorder begins to return to zero.

At the same time, contact N3B closes, energizing relay Q. Relay CR3 is energized through contacts Q1 and M1. This causes the motor on the pocket selecting device to operate until the contact brush with power from the stepping relay is lined up between the two halves of the contact segments, thus rotating the receiver disc until the pocket corresponding to the activity of the measured gold tube is lined up with the delivery tube.

(5) From 54 seconds to 58 seconds, contact N1B is closed, and solenoid S is operated by CR1 which is energized through N1B, P2, and M2. This rotates the upper half of the tubing in the ionization chamber until the hole lines up with the hole in the lower half and the gold tube drops into the pocket previously selected in the receiver disc.

The cycle is then repeated until all the gold implants are measured and sorted into the pockets corresponding to their activities.

The equipment described was completed and has been in continuous operation since 1941.

PLATE 87

FIGURE 1.—Control room for remote operation of radium emanation plant.

PLATE 87—Continued

FIGURE 2.—View of radium emanation plant through window shown in figure 1.

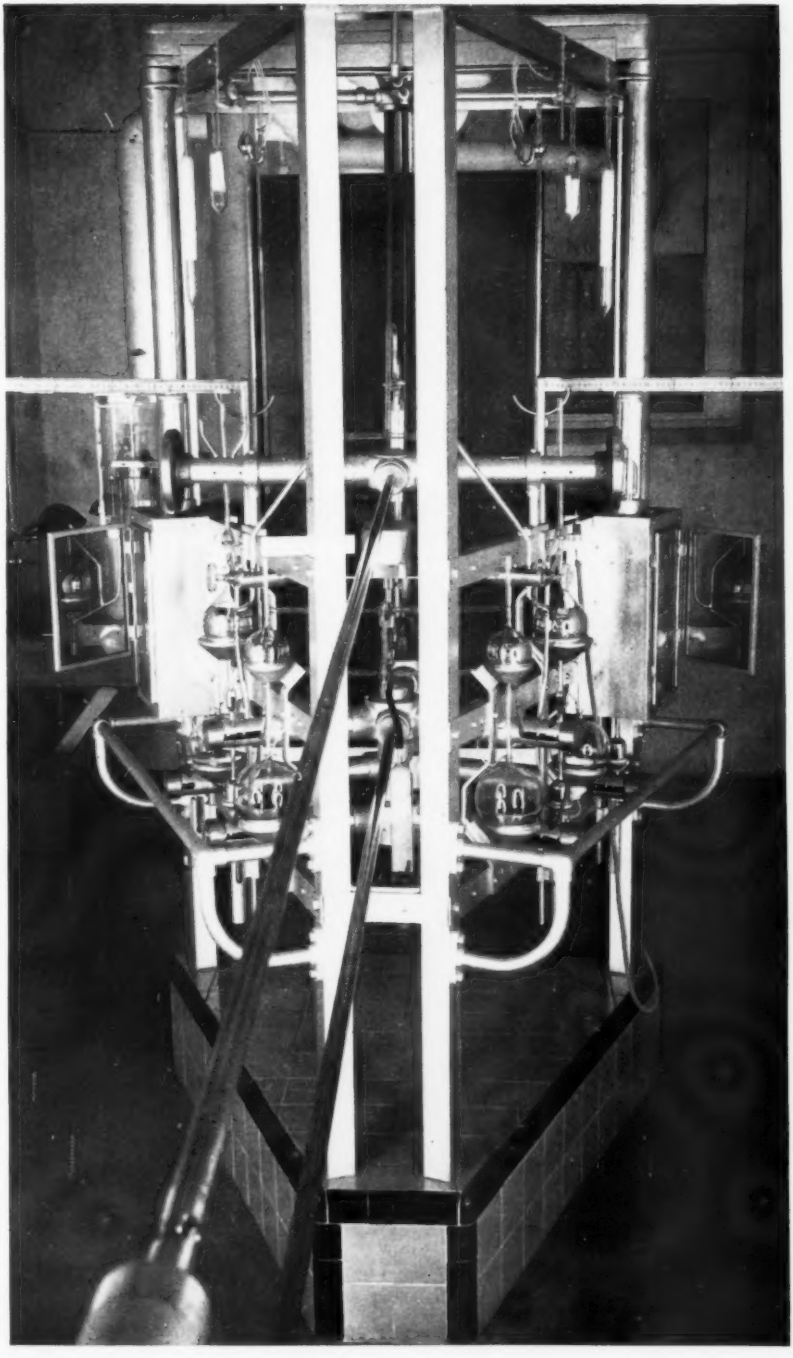


PLATE 88

FIGURE 3.—The Failla radioactivity recorder unit.

HEMATOPOIESIS IN THE SPLEEN OF TUMOR-BEARING HAMSTERS[1,2,3]

MARGARET A. KELSALL, Ph.D., *Department of Biology, University of Colorado, Boulder, Colo.*

Extramedullary myelopoiesis in the spleen, a source of confusion in the study of blood-forming tissues of mice (*1*, *2*) has been reviewed by Lang (*3*) and Barnes and Sisman (*1*) and has more recently been reported in mice having malignant tumors that were produced by various carcinogens (*4–7*) and in guinea pigs following injection of urine from patients with myelocytic leukemia (*8*). Splenomegaly has been reported in rats following injection of blood or urine from cancerous patients (*9*) or of a steroid obtained from human neoplasia (*10*).

The purpose of this work is to describe the progressive changes and significance of the extramedullary hematopoiesis that causes splenomegaly in Syrian hamsters having transplants of a mixed-cell sarcoma.

MATERIALS AND METHODS

The Syrian hamsters used in this work were from an inbred strain that has not been consistently brother-sister mated. The 119 hamsters were 79 to 315 days old and comprised 75 individuals of both sexes having subpannicular transplants of a mixed-cell, or polymorphous, sarcoma (Fig. 1) that was originally produced by 9,10-dimethyl-1,2-benzanthracene (*11*); 16 pregnant animals varying from 10 to 16 days post coitus and 5 lactating, 1 to 16 days, and 1 nonlactating, 20 days post partum; 8 on reduced stock diet of Purina dog checkers and barley without vitamin supplement for 16 to 23 days during which each lost 30 to 40 percent of its original weight; and 24 adult controls of both sexes and various ages.

Sections of the bone marrow imbedded by a special process (*12*) and of the liver and spleen of each animal were cut at 8 micra and stained with toluidin blue, Delafield's hematoxylin, Pappenheim's methyl green pyronin and by the Feulgen method for thymonucleic acid (*13*). Smears of spleen and bone marrow of each group were prepared with Wright's stain and by Graham's oxidase method.

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[1] Received for publication August 16, 1949.

[2] This work was supported by Beta Sigma Phi International Endowment Fund and a grant to Edward D. Crabb from the National Cancer Institute, National Institutes of Health, Public Health Service.

[3] The photomicrographs were made by Glenn E. Mills, University of Colorado Medical Center, Denver, Colo.

OBSERVATIONS

The development of extramedullary hematopoiesis, which does not normally occur in the spleen of adult hamsters (Fig. 2, *A*, *B*), was observed as foci of myeloblasts in the sinuses of the red pulp 16 days after the animals had been implanted with the tumor (Fig. 3, *B*). As extramedullary hematopoiesis progressed in the tumor-bearing hamsters, all stages of the erythrocytic and granulocytic series were present, and the poietic foci increased in size until the red and white pulp were indistinguishable (Figs. 2, *C*, and 3, *C*). Megakaryocytes (Fig. 2, *D*), which do not normally occur in the spleen of hamsters as they do in the spleen of mice (*14*), became numerous throughout the red pulp.

Only 2 of the 65 hamsters having a tumor duration over 15 days did not have extramedullary hematopoiesis in the spleen, but splenomegaly which was due to fibrosis (Fig. 3, *D*) was present in these 2 old animals, 268 and 315 days old.

Cells of both the erythrocytic and granulocytic series in the spleen were indistinguishable from similar cells in the bone marrow in nuclear and cytoplasmic characteristics, in content of thymonucleic acid (Fig. 3, *F*, *G*) and also in the number and distribution of oxidase granules (Fig. 3, *E*). No one stage of either the erythrocytic or granulocytic series predominated.

Extramedullary hematopoiesis occurred in the liver of 18 of the 68 tumor-bearing hamsters having hematopoiesis in the spleen (table 1), and each of the 75 animals that received implants of the tumor had periportal infiltration of lymphocytes and plasmacytes similar to that previously described in hamsters implanted with this sarcoma (*15*). However, extramedullary hematopoiesis did not occur in the thymus gland, peripheral lymph nodes, or intestinal lymphoid tissue of any animal.

Transplantation of hematopoietic tissue from the spleen of two and from the liver of four tumor-bearing hamsters did not take in hosts of the same inbred line. In addition, injection of heart blood from a donor having multiple metastasis of the sarcoma and splenomegaly due to hematopoiesis did not produce tumors or extramedullary hematopoiesis in four hosts.

Differential leukocyte counts on blood from the jugular vein of hamsters having splenomegaly following transplantation of the sarcoma showed a significant increase from 20.5 percent polymorphonuclear leukocytes for 4 controls and 31.9 percent for 6 pregnant hamsters to 67.8 percent for 16 tumor-bearing hamsters (*16*). Increase in the percentage of polymorphonuclear leukocytes was accompanied by a slight leukocytosis of 9,213 for the mean of 16 tumor-bearing hamsters compared with a mean of 5,358 in 6 pregnant hamsters of this inbred line (*16*). The mean

TABLE 1.—*Relation of hematopoiesis in the spleen and liver of sarcoma-implanted, pregnant, lactating, partly starved, and control hamsters*

Groups	Duration	Animals used	Spleen		Liver	
			Enlarged	Hematopoiesis	Periportal infiltration	Hematopoiesis
	Days	*Number*				
Sarcoma	1–15	5	0	0	5	0
	16–25	30	30	30	30	5
	26–50	35	35	34	35	10
	51–100	5	5	4	5	3
Pregnant	10–16	16	15	15	4	0
Lactating	1–16	5	5	5	3	0
Starved	16–23	8	0	0	0	0
Normal	----	24	0	0	0	0

of the total white cell count in the jugular blood of tumor-bearing hamsters is slightly higher than that obtained on cardiac blood in normal anesthetized hamsters (7,870 for females 2–4 months old to 8,560 for males and females over 9 months old (*17*).

Inanition was not responsible for hematopoiesis in the tumor-bearing hamsters because the 8 normal animals on the reduced diet for 16 to 23 days, during which 30 to 40 percent of original body weight was lost, did not have hematopoietic foci in the spleen (Fig. 3, *A*). In contrast, the spleens of these animals were slightly smaller than in the controls (Fig. 2, *A*) due chiefly to depletion of lymphoid tissue, an effect of starvation that is generally recognized.

Pregnancy produced the same changes in the spleen as the sarcoma (Fig. 2, *E, F*); however, the hematopoietic foci were well developed by the tenth day after mating, 5 days earlier than that following implantation of the sarcoma (table 1). Erythropoiesis and granulopoiesis in the spleen of the pregnant and lactating hamsters were similar to these processes in the tumor-bearing animals until 15 days post partum when the spleen began reverting to its normal size and structure and was similar to that of normal hamsters by the twentieth day post partum.

Although extramedullary hematopoiesis in the spleen was as extensive in the pregnant animals as in this organ of tumor-bearing hamsters, hematopoiesis did not occur in the liver of any pregnant or lactating animal; however, periportal infiltration of lymphocytes and plasmacytes was present in 4 of the 16 pregnant animals and in 3 of the 6 of the lactating group (table 1), but the amount of infiltration was very slight compared with that occurring in the liver of the tumor-bearing hamsters.

DISCUSSION

The red marrow in the long bones of normal hamsters, as in mice (*18*), occupies the entire marrow cavity; therefore, whenever need for an increase in hematopoietic cells occurs, such as during tumor growth or in pregnancy, the spleen probably takes over this function more readily than in those animals in which the red marrow is limited to only a small portion of the bone cavity and in which the yellow marrow becomes the first site for increased blood cells (*3*). Neither granulopoiesis nor erythropoiesis was observed in spleens of control hamsters in this strain in contrast to the frequent occurrence of nonmalignant hematopoiesis occurring in some older mice of several strains (*2*).

The presence of extramedullary hematopoiesis in tumor-bearing hamsters is in accord with the reports of myelopoiesis in the spleen of normal mice (*6*) and in some, but not all, mice having tumors induced by 9,10-dimethyl-1,2-benzanthracene (*4*) or by 1,2,5,6-dibenzanthracene (*5*).

SUMMARY

Erythropoiesis and granulopoiesis are the causes of splenomegaly in male and female hamsters having transplants of a mixed-cell sarcoma for over 15 days and also for the splenomegaly that occurs in normal hamsters during the period from the tenth day post coitus to the fifteenth day post partum.

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PLATE 89

FIGURE 1.—Histologic characteristics of the mixed-cell sarcoma used in this work. *A* and *B*, Primary implants. × 400. *C*, Metastasis to the right axillary lymph nodes. × 400. *D*, Metastasis to the pancreas Aselli. × 550. *E*, Metastasis to the kidney. × 400. *F* to *K*, Cellular variations to show the anaplastic nature of this sarcoma. × 1,200.

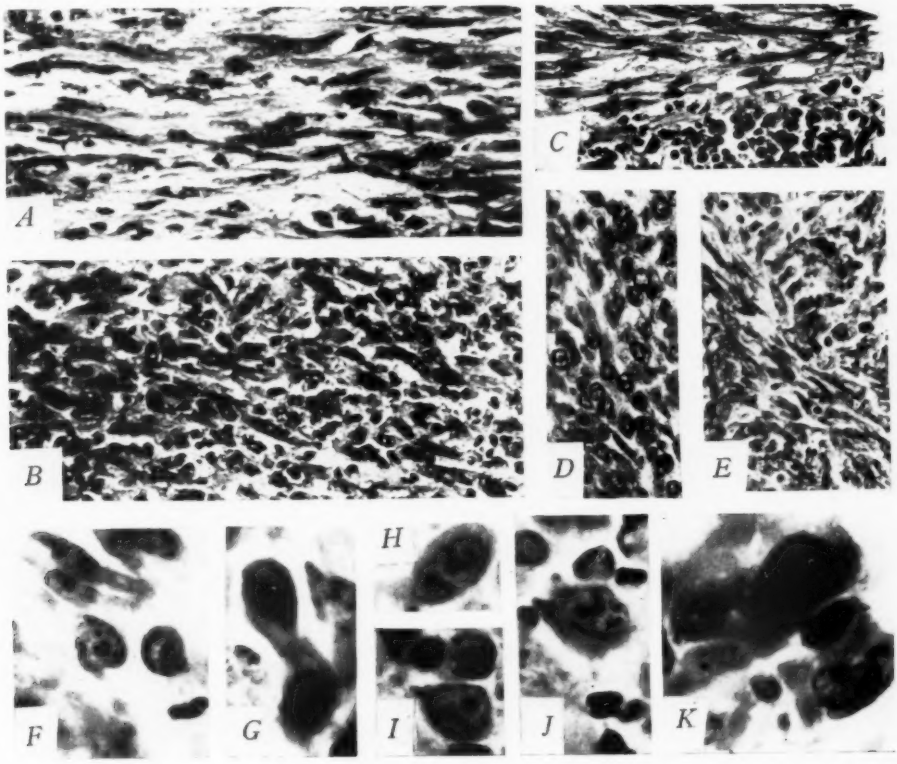
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PLATE 90

FIGURE 2.—*A*, Distribution of lymphoid tissue, the dark central areas, in the spleen of a control female 187 days old. Toluidin blue. × 33. *B*, Smear of red pulp of animal in *A* to show predominance of lymphocytes. Wright's. × 400. *C*, Depletion of lymphoid areas and foci of myeloblasts throughout enlarged spleen of a 190-day-old female having a transplanted sarcoma for 52 days. Toluidin blue. × 33. *D*, Megakaryocyte in smear of red pulp from same hamster as *C*. Wright's. × 550. *E*, Dark areas of hemopoiesis and remaining lymphoid tissue in enlarged spleen of a 175-day-old hamster 12.5 days post coitus. Toluidin blue. × 33. *F*, Megakaryocyte and myeloblasts in smear of red pulp from same pregnant female as *E*. Wright's. × 400.

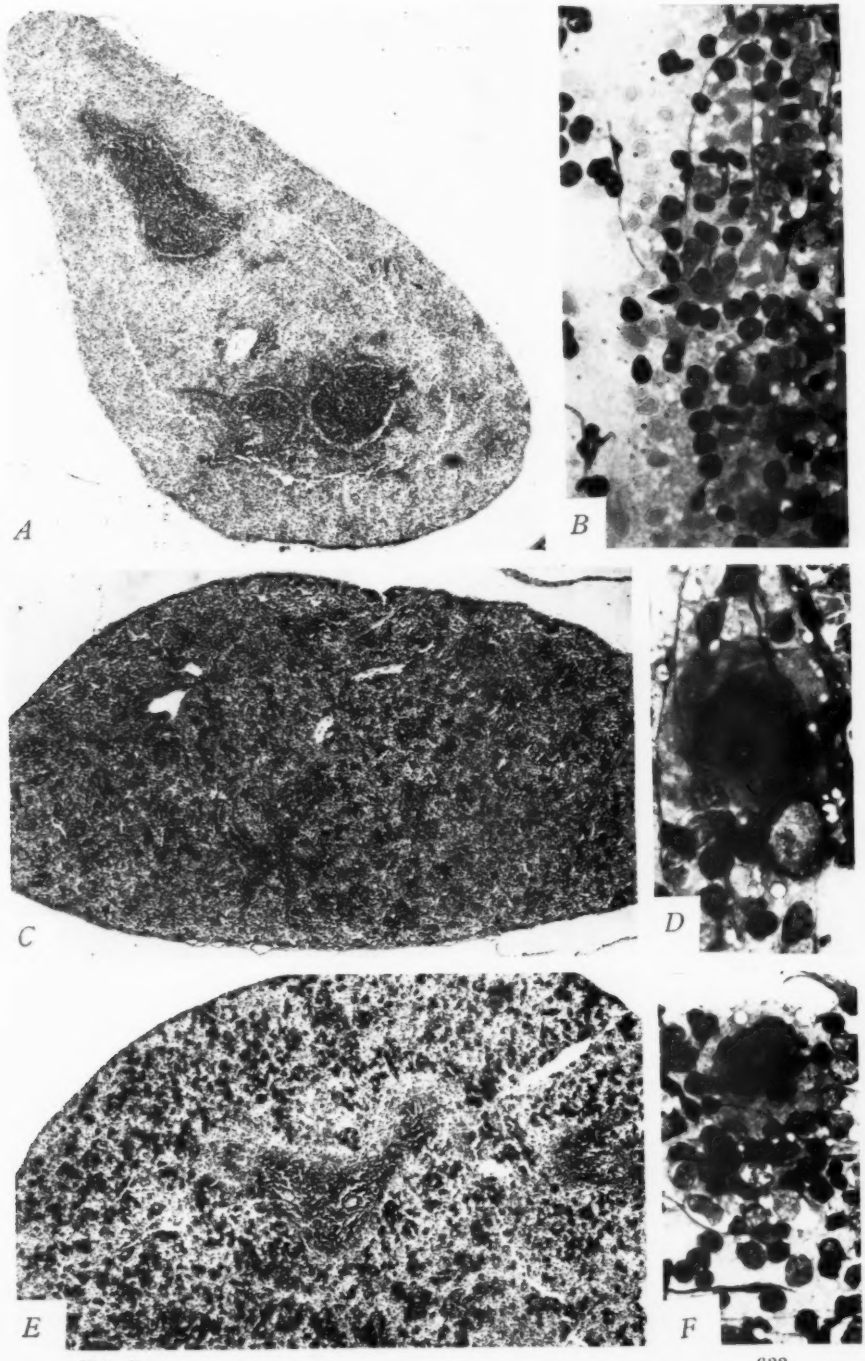
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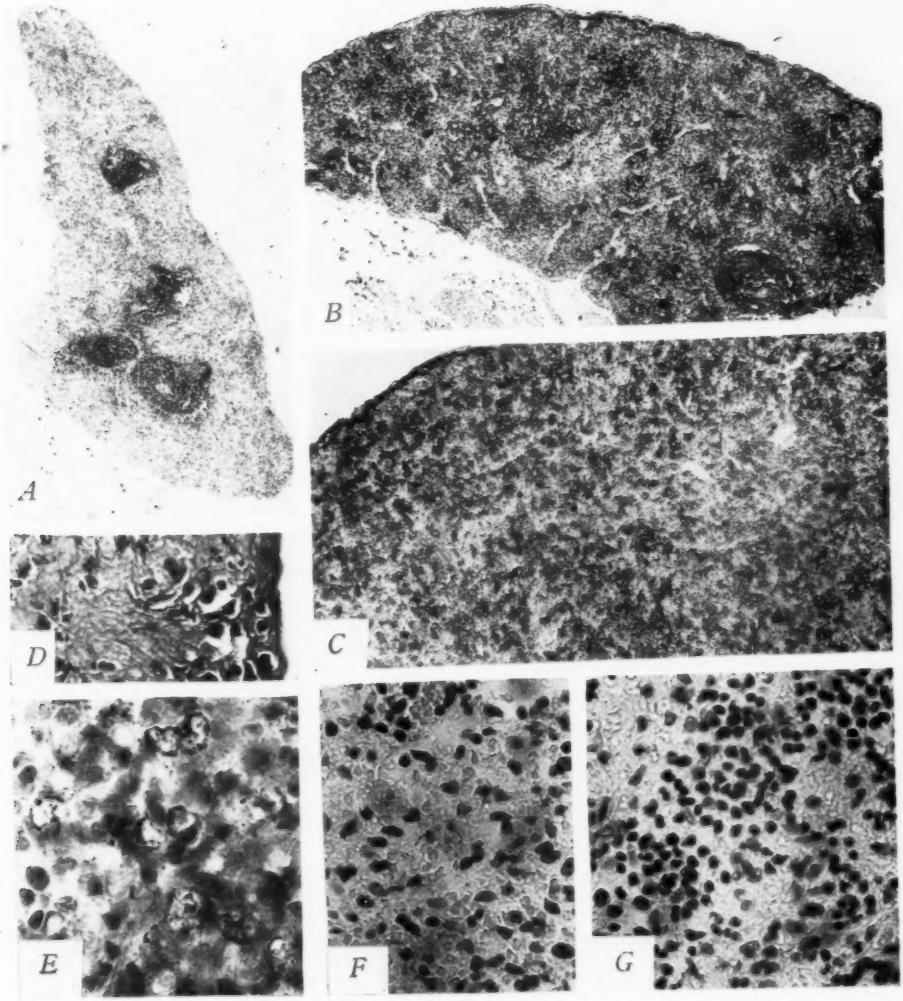
PLATE 91

FIGURE 3.—*A*, Depletion of lymphoid tissue and absence of hematopoiesis in the spleen of a 225-day-old hamster on reduced diet for 17 days with 33.5 percent loss of original weight. Toluidin blue. × 33. *B*, Spleen of a hamster, 213 days old, 16 days after implantation of the sarcoma. Toluidin blue. × 33. *C*, Spleen of hamster, 191 days old, that had a transplant of a sarcoma for 77 days. Toluidin blue. × 33. *D*, Fibrosis in the spleen of a hamster 315 days old and having a transplant of the sarcoma for 28 days. Delafield's hematoxylin and eosin. × 400. *E*, Oxidase granules in myelocytes in smear of the spleen of a 71-day-old hamster 13 days post coitus. Graham's oxidase. × 550. *F* and *G*, Sections of cortex of spleen of control hamsters (*F*) and of one having a transplant of the sarcoma for 72 days (*G*) to show thymonucleic acid in hematopoietic foci. Feulgen. × 300.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CATECHOLASE AND CATECHOLASE-INHIBITOR ACTIVITY OF HUMAN BLOOD SERA [1]

BERNARD SHACTER, Ph.D., and MICHAEL B. SHIMKIN, M. D., *Laboratory of Experimental Oncology, National Cancer Institute, National Institutes of Health, Public Health Service, and the University of California Medical School, San Francisco, Calif.*

There are numerous indications that the proteins of human serum and plasma are affected both quantitatively and qualitatively by the presence of certain disease states. Several recent reviews (*1, 2, 3*) have summarized and discussed such variations. One of the differences which may occur is a change in sulfhydryl content. Waldschmidt-Leitz (*4*) reported that the activity of glyoxalase, which is dependent on the presence of sulfhydryl groups, was enhanced to a greater extent on the addition of blood serum from normal individuals than from patients with cancer. A deficiency in the sulfhydryl content of serum from patients with cancer was indicated by Brdička (*5*) through polarographic analysis, while Black (*6*) suggested that the decrease in methylene blue reducing power of serum or plasma from patients with cancer, reported by himself (*7*) and by Savignac (*8*) was due to a deficiency of sulfhydryl groups.

While investigating the properties of serum catecholase and of catecholase inhibitor [2], it was observed that sera from normal subjects initially inhibited catechol oxidation for a longer time than sera from patients with cancer and several other diseases. Since the inhibitory capacity of serum was apparently due to the presence of a sulfhydryl component, further evidence was afforded of the decrease in sulfhydryl content of serum in patients with several disease states. This report describes the activity of catecholase and of catecholase inhibitor in sera from 128 individuals.

MATERIALS AND METHODS

Blood was drawn from the antecubital vein, generally before breakfast; in some instances, the blood was collected approximately 4 hours after the preceding meal. Serum was separated after normal clotting had occurred.

[1] Received for publication August 29, 1949.

[2] SHACTER, B.: Serum catecholase: Properties of the enzyme and nature of an endogenous inhibitor (In preparation.)

Catecholase and inhibitory activity of the serum was determined manometrically by measurement of oxygen uptake in the Warburg apparatus at 37° and pH 7.4. The pH of the serum was first brought back to the physiological range by neutralization of excess bicarbonate with hydrochloric acid. One ml. of serum and 0.25 ml.[3] of 0.1N HCl were added to the main chamber of the Warburg vessels, and 0.2 ml. of 10-percent potassium hydroxide was placed in the central well. The flasks, attached to lightly greased manometers, were equilibrated in the Warburg bath at 37° for 1 hour.

Following the neutralization equilibration, the flasks were removed from the bath, and 1 ml. of 0.5M phosphate buffer and distilled water to bring the volume to 2.5 ml. were added to the main chamber. Control flasks contained water and buffer. A catechol solution, containing 8 mg. per ml. in 0.2M phosphate buffer was freshly prepared, and 0.5 ml. was added to the side arm. The flasks with attached manometers were equilibrated in the bath for 10 minutes, the stopcocks closed, zero readings were taken, and the side arm contents were then tipped in. Readings were taken at intervals for at least 2 hours. The flasks were then removed from the bath, and the pH of the main chamber contents was determined with a glass electrode.

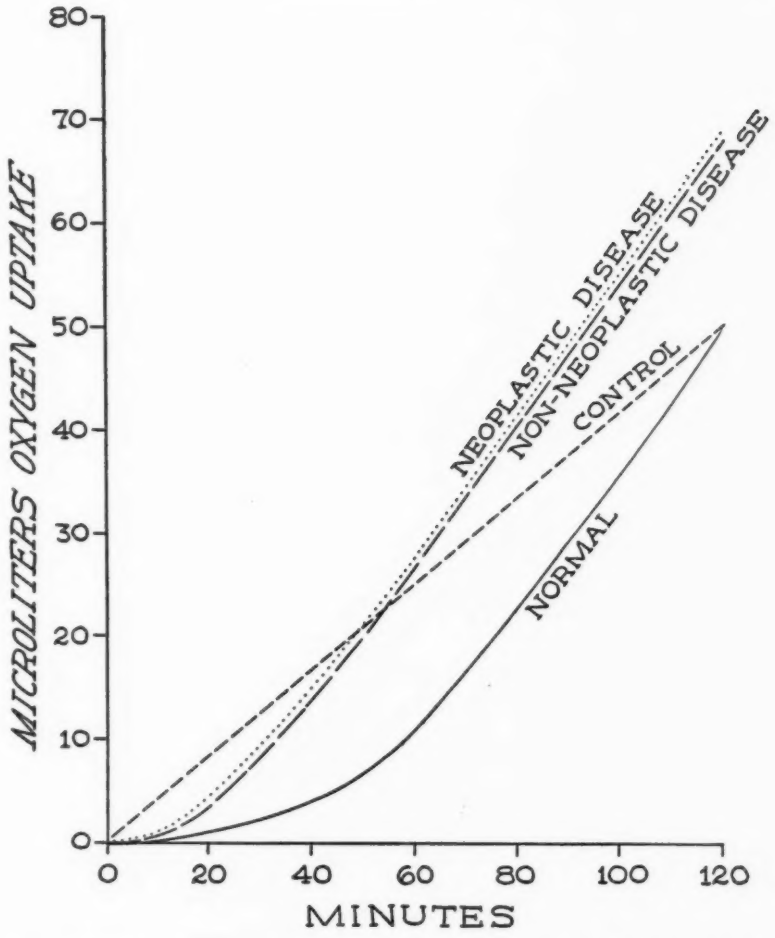
RESULTS

Typical curves of oxygen uptake as a function of time are shown in text-figure 1. After addition of catechol to serum there is an inhibitory period, in which the rate of oxygen uptake is retarded. This is followed by an accelerative period during which the oxidative rate reaches a maximum. The rate of uptake then remains constant for several hours. The inhibitory time, obtained by extending the linear portion of the curve to the time axis, was taken as a measure of catecholase-inhibitor activity. The difference between the maximum rates of oxygen uptake in experimental flasks and the rate in control flasks was used to estimate catecholase activity.

The activity of catecholase and its inhibitor was determined on sera obtained from 128 individuals as given in table 1. Of these, 35 were from individuals without evidence of disease, who ranged in age from 2 to 46 years. The remaining 93 sera were from patients with various clinical conditions. These included 7 normal gravid women, 20 to 35 years of age, with pregnancies ranging in duration from 2 to 8½ months.

Thirty-eight patients had various forms of neoplastic disease. All the patients, with the possible exception of one woman with Stage II carcinoma of the cervix, were clinically advanced cases beyond surgical or roentgenological probability of cure. The cases, on the other hand, were not terminal, and 21 are alive from 2 to 6 months following the determinations.

[3] Generally 0.25 ml. of acid was required for neutralization. In cases of acidosis or alkalosis, however, where the addition of this amount of acid gave a final pH outside the range 7.40±0.03, the determination was repeated with use of a smaller or larger amount of acid for neutralization.



TEXT-FIGURE 1.—Catechol oxidation in presence of normal and pathological sera.

The clinical conditions of the remaining 55 patients are recorded in table 1. In the group of 17 cases of cardiorenal-vascular disease, there were 7 patients with congestive heart failure. All 6 patients with tuberculosis had advanced lesions in the lungs.

The mean oxygen uptakes as a function of time for the normal subjects and patients with neoplastic and nonneoplastic diseases are presented in text-figure 1, and the values with the standard deviations are recorded in table 2. Control values show the rate of autoxidation of catechol. In all groups the mean oxygen uptake during the first 20-minute period was markedly inhibited as compared with the control uptake. The inhibition was significantly greater for the group of sera from normal indi-

TABLE 1.—*Activity of catecholase and catecholase inhibitor in human sera*

Condition	Subjects	Sex		Age	Catecholase activity			Inhibitory time		
		M	F		Range	Mean	S. D.[1]	Range	Mean	S. D.[1]
	Number			*Years*	*Microliters per hour*			*Minutes*		
Normal	35	17	18	2–46	9. 7–19. 7	16. 3	2. 6	33. 3–71. 9	47. 8	2. 9
Neoplastic disease	38	18	20	18–67	10. 6–21. 2	16. 4	3. 1	6. 1–44. 5	20. 3	8. 0
Carcinoma	16	6	10	31–67	10. 9–21. 2	------	------	6. 1–44. 5	------	------
Leukoses	14	7	7	24–66	10. 9–20. 9	------	------	11. 4–31. 4	------	------
Melanoma	4	3	1	38–67	10. 6–19. 7	------	------	8. 8–22. 0	------	------
Sarcoma	4	2	2	18–58	13. 9–19. 9	------	------	12. 7–33. 2	------	------
Cardiorenal-vascular disease	17	8	9	19–82	7. 0–22. 6	17. 2	3. 7	2. 6–30. 0	20. 0	8. 7
Congestive heart failure	7	2	5	32–79	7. 0–22. 6	------	------	2. 6–30. 0	------	------
Other	10	6	4	19–82	13. 1–20. 2	------	------	5. 2–29. 2	------	------
Pulmonary tuberculosis	6	2	4	41–50	9. 6–21. 4	17. 0	------	7. 7–17. 8	13. 0	------
Other diseases	25	13	12	5–79	6. 3–22. 9	16. 1	3. 6	12. 2–46. 0	26. 4	9. 4
Liver damage	3	2	1	45–56	11. 7–18. 4	------	------	13. 2–24. 1	------	------
Rheumatoid arthritis	3	------	3	18–31	12. 7–16. 5	------	------	14. 8–36. 9	------	------
Bronchial asthma	2	2	------	50, 57	18. 1, 20. 5	------	------	16. 1, 18. 1	------	------
Diabetes mellitus	3	1	2	41–65	14. 2–19. 9	------	------	21. 4–46. 0	------	------
Thyrotoxicosis	2	1	1	52, 68	14. 0, 18. 4	------	------	22. 1, 39. 4	------	------
Central nervous system	3	3	------	11–79	12. 3–22. 9	------	------	12. 2–31. 9	------	------
Unclassified	9	4	5	5–61	6. 3–19. 6	------	------	13. 3–38. 8	------	------
Pregnancy	7	------	7	20–35	12. 2–18. 4	15. 1	------	14. 1–28. 3	20. 9	------

[1] Standard deviation.

TABLE 2.—*Comparison of rates of oxygen uptake*

Group	Determinations	Microliters O_2 uptake in time interval											
		0-20 minutes		20-40 minutes		40-60 minutes		60-80 minutes		80-100 minutes		100-120 minutes	
		Mean	S. D.[1]	Mean	S. D.	Mean	S. D.	Mean	S. D.	Mean	S. D.	Mean	S. D.
Control	15	8. 2	1. 3	8. 5	0. 6	8. 5	0. 5	8. 5	0. 8	8. 3	0. 6	8. 1	0. 7
Normal	35	1. 1	1. 0	3. 2	1. 3	6. 8	3. 2	11. 9	2. 5	13. 1	1. 1	13. 8	1. 2
Neoplastic disease	38	4. 2	2. 4	10. 8	3. 2	12. 9	1. 6	13. 5	1. 5	13. 9	1. 1	13. 7	1. 1
Nonneoplastic diseases	48	3. 5	2. 9	10. 0	3. 3	12. 9	1. 8	13. 9	1. 4	13. 7	1. 5	13. 9	1. 3
Pregnancy	7	3. 5	------	10. 8	------	11. 8	------	13. 3	------	13. 1	------	13. 8	------

[1] Standard deviation.

viduals than for the groups of sera from patients with neoplastic diseases and other clinical conditions. The differences in oxygen utilization were most pronounced in the 20- to 40-minute time period. Marked inhibition of oxidation was still present with normal sera, while the inhibitory effect had been largely released with other sera and the oxidative rates now exceeded that of the autoxidation of catechol. After 60 minutes the disparity in rates between the normal and other groups decreased rapidly. The maximum rate of oxidation was approximately the same for all groups.

The histogram of text-figure 2 gives a detailed analysis of catecholase inhibitor activity. Of 38 patients with neoplastic disease, the inhibitory times of 3 overlapped into the range observed with normal sera. None of the sera from cardiorenal-vascular disease, tuberculosis, or pregnancy fell within the normal range.

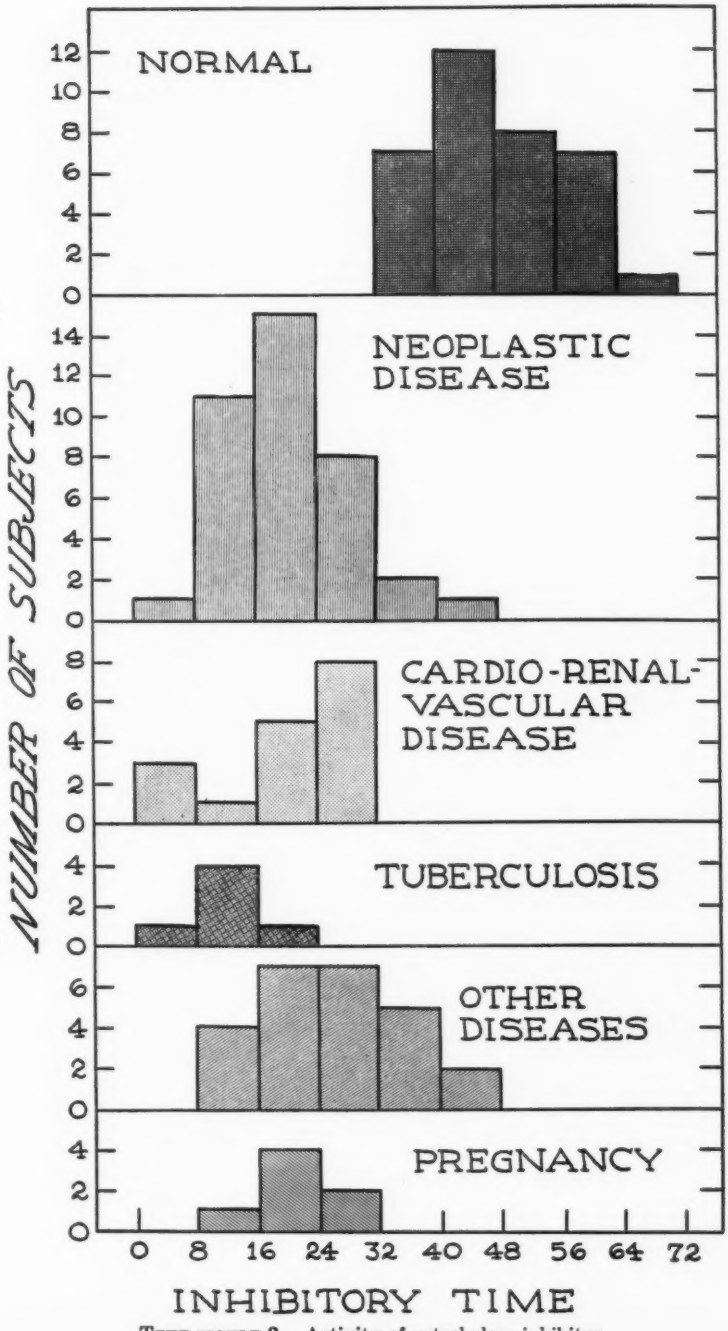
DISCUSSION

The results of the investigation show definite differences between normal human blood sera and sera obtained from patients with various clinical conditions in their capacity initially to retard the oxidation of catechol. It was shown [2] that the factor in serum responsible for this effect is probably a sulfhydryl compound. The data therefore indicate, by still another technique, that the sulfhydryl content of serum may fall with the development of a number of pathologic states, including cancer and allied diseases.

Most of the normal sera were obtained from individuals in the 20 to 35 year age range, while most of the patients were over 35 years old. No correlation between the inhibitory capacity and age could be established in any of the groups, and it is felt that age *per se* is not an important factor in the observed differences. There was no correlation between the red blood count or hemoglobin values and the initial inhibition of catecholase activity; in the cases where determinations were available on the albumin and globulin content of the serum, no correlation was apparent.

It should be clear from table 1 and text-figure 2 that no implication is possible regarding the use of this procedure in the diagnosis of cancer or other pathologic states, even in the advanced stages of disease utilized in the study. What can be concluded, on the basis of the data obtained, is that in certain diseases the serum loses part of its capacity initially to inhibit the oxidation of catechol, because of reduction in the content of effective sulfhydryl groups. The maximum catecholase activity, on the other hand, is approximately constant for all sera, normal or pathological.

It is interesting to postulate the mechanisms that may be involved in changes in the sulfhydryl content of sera in such apparently dissimilar conditions as pregnancy, neoplastic diseases, tuberculosis, congestive heart failure, and rheumatoid arthritis.



TEXT-FIGURE 2.—Activity of catecholase inhibitor.

Changes in the excretion of adrenal cortical steroid metabolites, denoting possible changes in adrenal cortical activity, have been noted in neoplastic disease (*9*), congestive heart failure (*10*), and pregnancy (*11, 12*), while certain forms of arthritis are apparently affected by adrenal cortical activity (*13*). Furthermore a reciprocal relationship apparently exists between cortical activity and sulfhydryl compounds. Conn *et al.* (*14, 15*) recently demonstrated that following the administration of adrenocorticotropic hormone to human subjects, a fall in blood glutathione occurs along with the glycosuria and hyperglycemia. Moreover, the diabetes in these subjects may be alleviated by administration of glutathione (*16*). It is suggested, therefore, that the shift in the sulfhydryl content of blood which occurs in pregnancy and in several disease states is at least partially brought about by a change in adrenal cortical activity.

West and Hilliard (*17*) recently reported the occurrence of abnormal levels of chymotrypsin and rennin inhibitors in the sera of patients with various clinical conditions as compared with normal individuals. The distribution of abnormal levels of chymotrypsin and rennin inhibitors in disease states followed closely the activity of catecholase inhibition in the same states. The two phenomena may, therefore, be related. West and Hilliard concluded that steroid hormones may be implicated also in chymotrypsin and rennin inhibitor activity.

SUMMARY

Catecholase and catecholase-inhibitor activity of human sera was determined in 35 normal individuals and 93 patients with neoplastic diseases and other clinical conditions. The maximum catecholase activity was approximately equal for all sera.

Sera differed in their capacity initially to inhibit catechol oxidation; the inhibitory capacity is due to a sulfhydryl compound. The inhibitory capacity was greatest in normal sera and was markedly diminished in pregnancy, pulmonary tuberculosis, cancer, congestive heart failure, and several other clinical conditions. These pathological states apparently produce, or are associated with, a reduction in the effective sulfhydryl content of blood serum.

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COMPARATIVE STABILITY OF THE AGENT OF CHICKEN TUMOR I IN PHOSPHATE AND BORATE BUFFERS DURING INCUBATION FOR 3 HOURS AT 37° C.[1]

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Preparatory to contemplated enzymatic studies on the agent of chicken tumor I (Rous Sarcoma) (*1*) involving incubation at a temperature of 37° C., it was desired to know whether the stability of the biological activity would vary significantly in different types of buffers that might be employed for controlling pH. The present investigation was for the purpose of comparing the relative biological activity of tumor-agent suspensions in phosphate buffers and in borate buffers after incubation for 3 hours at 37° C. In addition to the pH range within which the two buffer systems overlap, observations on biological activity were extended to include essentially the entire pH range obtainable with each type of buffer.

MATERIALS AND METHODS

Phosphate Buffers

Primary (KH_2PO_4) and secondary ($Na_2HPO_412H_2O$) phosphate solutions were prepared in 0.1 molar concentration, according to Clark (*2*), and mixed in the appropriate proportions required to give each desired pH. The molar concentration was twice that (0.05) desired in the final agent-buffer mixtures.

Borate Buffers

The borax and boric acid (+NaCl) solutions of Palitzsch were prepared as described by Clark (*2*). Various mixtures of these two solutions were then made up which gave the desired pH when diluted to a total salt strength of 0.1 molar.

pH Determinations

All determinations of pH were made at room temperature by means of a Macbeth Line-operated pH meter [2] equipped with a glass electrode. The accuracy of this instrument is stated to be 0.1 pH.

[1] Received for publication September 12, 1949.

[2] Manufactured by the Macbeth Corporation, New York.

Tumor-Agent Suspensions

In each experiment 10 gm. of frozen tumor tissue were allowed to thaw at room temperature and homogenized in 300 ml. of cold 0.9-percent NaCl solution by blending for 3 minutes in a Waring blendor. Most of the tissue debris and the heavier particulates were removed by centrifuging for 20 minutes at approximately 2,300 g in an angle head of an ordinary laboratory centrifuge. The supernatant was recovered and subjected to a short interval of centrifugation in a multispeed centrifuge [3] which was accelerated up to 18,000 g and then immediately cut off and allowed to come to rest gradually by means of a free-coasting device.[3] The middle third of the supernatant from this short, high-speed run was carefully pipetted out, and 72 ml. of it were subjected to further centrifugation at 18,000 g for 1 hour. The sediment resulting from this long run was recovered and resuspended in 40 (experiment 1) or 48 (experiment 2) ml. of cold distilled water. The suspension was cleared of larger aggregates and particulates by centrifugation for a short time at high speed in the same manner as described above. The supernatant from this, the final centrifugation, was carefully pipetted off without disturbing the sediment and was used in the experiment as the aqueous tumor-agent suspension. A temperature of 0°–8° C. was maintained at all times during preparation of the agent and throughout the experiment except during the designated time of incubation.

EXPERIMENTAL PROCEDURES

Two separate quantitative experiments were carried out in which estimates were made of the biological activity remaining in various buffer suspensions of tumor agent after incubation for 3 hours at 37° C. The relative activity was determined in each instance by comparison with an unincubated reference suspension which had been prepared from the same batch of tumor agent.

The buffers used in experiment 1 were phosphate solutions of pH 5.0, 6.0, 6.5, 7.0, 7.5, and 8.0 and borate solutions of pH 7.0, 7.5, 8.0, and 8.5. In experiment 2 the phosphate buffer solutions were of pH 6.5, 7.0, 7.5, 8.0, and 8.5, the last being composed almost entirely of the secondary phosphate and, therefore, not an efficient buffer. The borate buffer solutions employed in experiment 2 were of pH 6.5, 7.0, 7.5, 8.0, 8.5, and 9.0, the first of these being below the efficient buffer range. The concentration of all buffer solutions of both experiments was 0.1 molar.

The suspensions of tumor agent in the various buffers were made by adding 3 ml. aliquots of aqueous tumor agent suspension to equal quantities of 0.1M buffer solution. The molarity of the final mixtures was, therefore, 0.05 with respect to buffer salts. Immediately after their preparation, the suspensions of tumor agent in buffer were placed in a closed water bath where they were held at 37° C. for 3 hours, being stirred at approxi-

[3] Multispeed attachment, head no. 295, of an International Refrigerated centrifuge, Model PR-1.

mately 15-minute intervals during this time. After the period of incubation, they were transferred to an ice water bath and a 1.5 ml. sample of each was taken for bioassay. The remainder of each suspension was then allowed to come to room temperature for a pH determination.

In each experiment a reference suspension for use in the bioassays was prepared from the same batch of aqueous tumor-agent suspension as that employed in the buffer suspensions. It contained normal rabbit serum [4] as a stabilizing factor and was kept at a low temperature (0°–8° C.) at all times in order to maintain, insofar as possible, the initial level of biological activity of the tumor agent. The reference suspensions were prepared by diluting 10 ml. of aqueous tumor-agent suspension with an equal quantity of 0.1 M phosphate buffer, of pH 7, containing 4 percent, by volume, of normal rabbit serum. The final molarity of buffer salts was, therefore, 0.05, and the final concentration of serum was 2 percent by volume. The concentration of tumor agent was the same in the reference suspension and in all buffer suspensions of the same experiment.

The samples taken for bioassay, both of the reference suspensions and of all buffer suspensions (unknowns), were immediately diluted $\frac{1}{10}$ and $\frac{1}{100}$ with a diluent consisting of 0.05 M phosphate buffer, of pH 7, to which had been added 2 percent, by volume, of normal rabbit serum. The diluted materials were inoculated into chickens for bioassays as described in the following section. All materials injected into chickens contained, therefore, approximately 2 percent normal rabbit serum and had an approximately neutral reaction at the time of injection.

Bioassays

The bioassay procedure was an adaptation of the method described by Bliss and Marks (*4*) for the assay of insulin. Its application to assays of the agent of chicken tumor I was illustrated and discussed in a previous report (*5*). For each bioassay determination, 2 doses of the unknown and 2 of the reference suspension were inoculated subcutaneously, at different sites, into each of 40 chickens. Each bioassay was therefore based upon 160 unit observations. The 2 doses of each material consisted of 0.2 ml. quantities of the $\frac{1}{10}$ and $\frac{1}{100}$ dilutions prepared as described in the preceding section. The 40 inoculations of each dose were arranged on chickens and sites according to a 4×4 Latin square design.

As in the procedure previously illustrated (*5*), the slopes of the dose-response curves for all bioassay determinations within the same experiment were averaged, and the average value was used for computing the potency estimate for each of the separate unknowns. This procedure was

[4] The serum of various animals, as well as bacterial broth culture media, is widely used as protective agents in work with labile viruses, particularly in the diluents used for titrating biological activity. Serum has also been widely used in studies on the highly labile virus-like agent of chicken tumor I. Gye and Purdy (*3*) found that, of sera tested, horse, rabbit, goat, and fowl sera were all effective in prolonging the infectivity of filtrates. They suggested that serum acts "simply as a colloid damping the rapidity of chemical reactions." Experiments carried out in this laboratory (unpublished) confirm the value of rabbit serum for prolonging the period over which partially purified suspensions of the agent of chicken tumor I remain highly active; quantitative data are lacking, however, which would permit an accurate discussion of the degree of protection to be expected over a given time.

followed because of the relatively flat slope of the dose-response relationship (*6*) and the resultant fact that individual estimates of slope based upon only 40 chickens (160 sites) are subject to relatively large errors and are not sufficiently accurate to be used as separate estimates. The use of wider dose intervals to increase the accuracy of the slope estimates within groups of 40 chickens was precluded by the fact that when the interval between the strongest and weakest doses becomes too wide, death results from tumors produced by the stronger doses before the weaker ones have had full opportunity to produce their effects (*6*).

In a similar manner, and for the same reasons, the errors of the slope estimates were averaged over all bioassay determinations of the same experiment, and the average value was employed in computing the final error of each of the separate potency determinations. The limits of error estimated in this manner are not presented as exact confidence limits but as useful approximations which should allow accurate judgment of the data except in those cases where differences, or variations, are on the borderline of significance. The consideration of more exact statistical treatment is withheld pending further investigation.

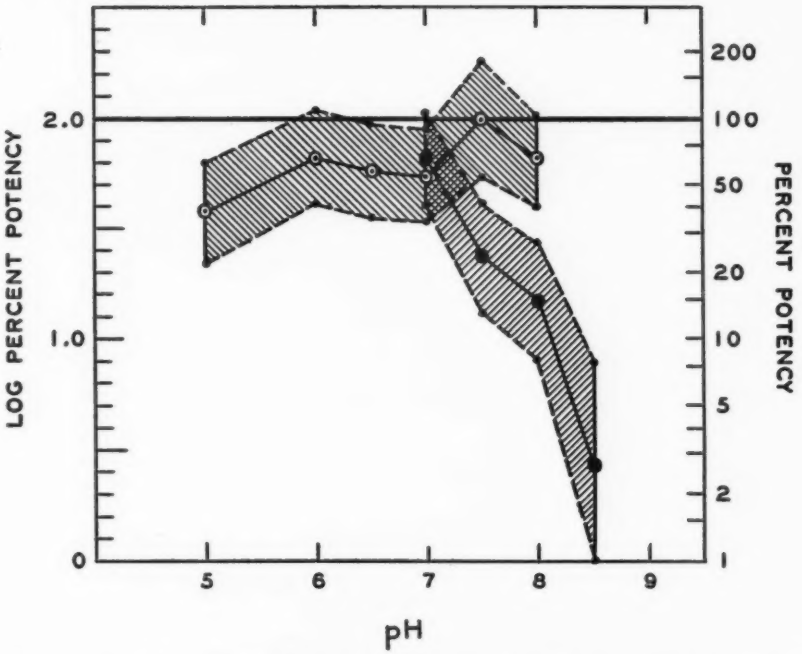
In only 1 of the 21 bioassays of the present investigation was there a significant departure from parallelism of the dose-response curves for unknown and reference suspensions. In this instance, material 10 of experiment 1 (see table 1), the common slope for the 2 materials was significantly lower than the other 9 estimates of the same experiment; this slope value and its error were, therefore, omitted in deriving the averages which were employed as described above for the analysis of data of experiment 1.

RESULTS

Table 1 and text-figures 1 and 2 show the relative potencies of the various buffer suspensions of tumor agent after they had been incubated for 3 hours at 37° C. The pH of the suspensions taken after the period of incubation differed in only one case from the initial pH by more than the limits of accuracy of the pH meter. In this instance (borate buffer pH 6.5 of experiment 2), the pH was initially below the efficient buffer limit, but, even so, the change, from pH 6.5 to 6.9, was not pronounced. The bioassay results are given both in terms of percent and log percent, the latter having been used for the biomathematical analyses and for their graphic representation. The plotted points of text-figures 1 and 2 represent the data of experiments 1 and 2, respectively, the results obtained with the different types of buffer being indicated by the different type symbols. The shaded bands of the figures enclose the limits of error corresponding to ± 1 standard error unit; they are included simply as an aid to the visual appraisal of the trends and secondary fluctuations of the plotted points, which are subject to relatively wide chance variations, and are not intended as graphic substitutes for statistical analyses.

The data of the two experiments are consistent in showing that although there were no essential differences between results obtained with the two

types of buffer at pH 6.5 and 7.0, real differences occurred at pH 7.5 and above. The phosphate buffer suspensions showed no marked dependency of potency on pH within the range tested, but the potency of the borate buffer suspensions decreased progressively as the pH was increased. The negative regression of potency on pH was statistically significant, $P=0.026$ in both experiments, thus indicating that there was a real inhibiting or inactivating effect of the borate ions on biological activity of the agent of chicken tumor I. Although the borate effect was



TEXT-FIGURE 1.—Relative potencies at various levels of pH of tumor-agent suspensions in phosphate and borate buffers after incubation for 3 hours at 37° C.
Open circles—phosphate buffer suspensions;
Closed circles—borate buffer suspensions;
Shaded bands—error zones limited by ±1 standard error unit;
Data of experiment 1.

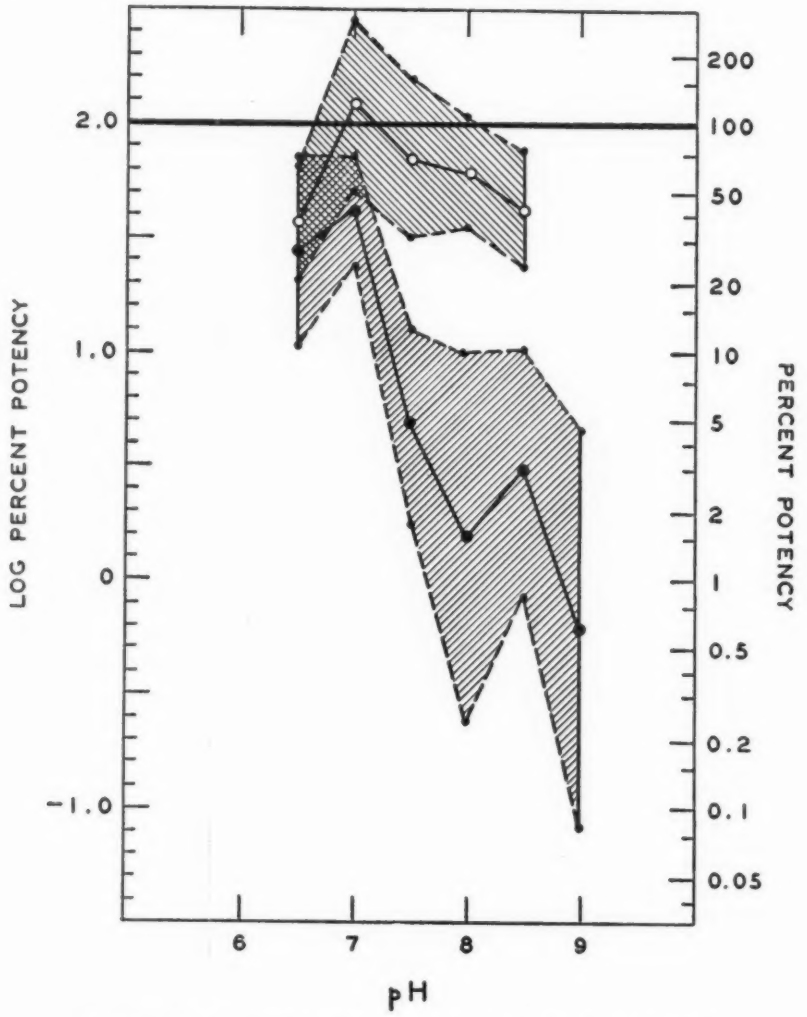
dependent upon pH, it was of much greater importance than the effect of pH itself within the range represented. Whether these findings represent an *in vitro* inactivation of the agent or whether the small amounts of borate (0.005 or 0.0005 molar), present in the diluted materials used for the chicken inoculations, locally inhibited the action of the agent on the living cells cannot be determined from the present studies. It is clear, however, that alkaline borate buffers are much less satisfactory than phosphate buffers for use as suspending media in biological studies on the

TABLE 1.—*The potencies of incubated suspensions of tumor agent in various buffers in relation to reference suspensions*

Experiment No.	Material No.	Type of buffer	pH		Biological activity relative to reference suspension			
			Of added buffer	Of mixture after 3 hours at 37° C.	Log percent potency	Standard error of log percent	Percent potency	Standard error of percent
1	1	Phosphate	5.0	5.23	1.576	±0.230	37.7	+26.3 −15.5
	2	do	6.0	6.06	1.823	±.210	66.6	+41.3 −25.5
	3	do	6.5	6.55	1.763	±.214	57.9	+36.8 −22.5
	4	do	7.0	7.00	1.741	±.209	55.1	+57.1 −21.1
	5	do	7.5	7.47	2.002	±.260	100.4	+82.5 −45.3
	6	do	8.0	7.95	1.808	±.202	64.3	+38.1 −23.9
	7	Borate	7.0	7.10	1.825	±.200	66.8	+39.0 −24.6
	8	do	7.5	7.49	1.369	±.246	23.4	+17.8 −10.1
	9	do	8.0	8.06	1.176	±.275	15.0	+13.3 −7.0
	10	do	8.5	8.42	.444[1]	±.447[1]	2.8[1]	+5.0[1] −1.8
2	1	Phosphate	6.5	6.68	1.555	±.245	35.9	+27.2 −15.5
	2	do	7.0	7.02	2.079	±.373	120.1	+163.2 −69.2
	3	do	7.5	7.55	1.839	±.361	69.1	+89.5 −39.0
	4	do	8.0	7.93	1.790	±.243	61.6	+46.3 −26.4
	5	do	8.5	8.33	1.623	±.256	42.0	+33.7 −18.7
	6	Borate	6.5	6.90	1.439	±.414	27.5	+43.8 −16.9

7	do	7.0	7.14	1.615	±.240	41.2	+30.4 −17.5
8	do	7.5	7.60	.680	±.423	4.8	+7.9 −3.0
9	do	8.0	8.22	.188	±.805	1.5	+8.3 −1.3
10	do	8.5	8.41	.476	±.542	3.0	+7.4 −2.1
11	do	9.0	8.85	−.206	±.871	.62	+4.0 −.54

[1] Bioassay data showed a significant departure from parallelism of the curves for reference and buffer suspensions; see text, p. 650.



TEXT-FIGURE 2.—See legend for text-figure 1. Data of experiment 2.

agent of chicken tumor I, and that the choice of buffer is an extremely important consideration in quantitative experiments designed to study the nature and mode of action of the agent of chicken tumor I.

DISCUSSION

The significance of the inactivating effect of alkaline borate solutions on the agent of chicken tumor I cannot be stated at the present time. Borates are known to form complexes with many polyhydroxy compounds of low molecular weight. These complexes are more readily obtained from glycols with the *cis* configuration than with the *trans* isomers. Because of this ability to form complexes, boron inhibits the oxidation of glucose by hypoiodite, as shown by Myrbäck and Gyllensvärd (*7*). Militzer (*8*) found that borate inhibited the chemical oxidations of 5-keto-D-gulonic acid, dehydro-l-ascorbic acid and 2,3-diketo-L-gulonic acid by cupric ions, methylene blue and molecular oxygen. This inhibition by borate was most effective at an alkaline pH. Under the same conditions the oxidations of sulfhydryl groups and of the enediol of ascorbic acid were not inhibited by borate.

Borates have also been found to inhibit several physiological reactions. Kisch (*9*) reported that boric acid and borates in concentrations of 0.0002M inhibited the respiration of mammalian tissues, (kidney, heart, and liver). Less concentrated solutions stimulated respiration and both the inhibition and stimulation were less marked at pH 6.9 than at pH 7.4–8.2. Zittle (*10*) found that the phospho-diesterase and the phospho-monoesterase of calf intestinal mucosa were reversibly inhibited approximately 50 percent by 0.01M borate. Because these enzymes participate in the catabolism of nucleic acid, Zittle suggested the possibility that borate may influence the pathological processes involved in cellular multiplication.

SUMMARY

The stability of the agent of chicken tumor I (Rous sarcoma) at 37° C. was studied at different levels of pH in phosphate and borate buffers. Suspensions of tumor agent in phosphate buffers of pH 5.0 to 8.5 and borate buffers of pH 6.5 to 9.0 were incubated for 3 hours at 37° C., after which samples were taken for bioassays. Significant differences were found between the potencies of phosphate and borate buffer suspensions of the same pH within the alkaline pH range, but no appreciable differences were observed at neutral or slightly acid reactions.

One table and two charts are presented which show the relative stability at 37° C. of the agent in phosphate and borate buffers at various levels of pH.

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LIVER-GLUCURONIDASE ACTIVITY OF INBRED MOUSE STRAINS[1,2]

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In recent years there has been increasing interest in the possible relationship between enzymes and the development of spontaneous and induced tumors. Studies of hepatic enzyme activity in inbred mice without tumors have been reported by several investigators: xanthine dehydrogenase (*1–3*), esterase (*4–6*), catalase (*2, 3, 7, 8*), cystine oxidase (*9*), arginase (*2*), acid phosphatase (*2*), alkaline phosphatase (*2*), thymonucleodepolymerase (*2, 3*), ribonucleodepolymerase (*2*), and amylase (*3*).

Figge and Strong (*1*) found less xanthine dehydrogenase activity in the livers of C3H mice than in JK mice. However, Greenstein (*2*) could not demonstrate differences when C3H mice were compared to the C, A, and dilute brown strains. Greenstein (*8*) found the liver-catalase activity of the C57 black strain about half that of the C3H, A, C, dilute brown, I, or Y strains. Measurements of the other enzymes listed have shown no differences among several inbred strains.

The present investigation was undertaken to determine the liver-glucuronidase activity of various inbred strains of mice and to compare this activity with the reported incidence of spontaneous mammary and hepatic tumors (*10–13*).[3] The relationship between glucuronidase and estrogenic hormones (*14, 15*) suggested the possibility that differences might exist.

MATERIALS AND METHODS

Mice of the C3H, A, dba, C, and C57 black strains maintained at the National Cancer Institute were employed in the experiments. The

[1] Received for publication September 23, 1949.

[2] The authors wish to acknowledge the helpful suggestions of Dr. Emanuel B. Schoenbach, M. D., consultant Clinical Research Unit, National Cancer Institute, and Dr. A. Goldin in the planning and preparation of these studies. Dr. W. E. Heston, principal geneticist, National Cancer Institute, kindly provided the C3H (Heston) mice employed.

[3] Law, Lloyd, National Cancer Institute: Personal communication.

animals were segregated as to sex and maintained on an ad libitum diet of Purina laboratory chow and water. Ten of the C3H (Heston) mice had been foster-nursed on C57 black mothers free of the mammary-tumor inciter (milk factor). Otherwise, the high-tumor strains, C3H (Heston), and A, possessed the milk factor. The animals selected weighed 18–25 gm. and were without gross evidence of tumor when killed.

The glucuronidase assays were carried out by a modification of the method of Talalay (*16*) using phenolphthalein glucuronide as the substrate. Animals were killed individually by cervical dislocation. A portion of the liver was removed and weighed as rapidly as possible on a microtorsion balance. For all strains except the C3H, portions of liver of about 100 mg. liberated sufficient phenolphthalein for accurate measurement. Because of the low-enzyme activity in the C3H mice, 300 mg. of liver were employed.

Each piece of liver was homogenized in 5.0 ml. of distilled water in an ice-bathed Potter-Elvehjem glass homogenizer. Aliquots of the whole (uncentrifuged) homogenate were used instead of the supernatant fluid recommended by Fishman. This was done because in initial experiments it was found that the sediment of centrifuged homogenate showed moderate glucuronidase activity of varying degree. Uniform results were obtained with the whole homogenate. After incubation at 37.5° C. for 1 hour with 0.0005M phenolphthalein glucuronide (pH 4.5), the reaction was stopped, and the red color was developed by the addition of alkaline glycine buffer of pH 10.45. The tubes were rapidly centrifuged for 5 minutes, decanted into colorimeter tubes and read against the control at 550 in a Coleman Model 6A spectrophotometer. Each homogenate was assayed in duplicate.

The liberated phenolphthalein was measured by comparison of the galvanometer deflection with a standard phenolphthalein curve. Results are expressed as micrograms of phenolphthalein liberated per milligram of moist tissue after incubation for 1 hour.

RESULTS

The livers of 115 animals of 5 strains were assayed for glucuronidase activity. Variation between the duplicates was less than 5 percent. The results are summarized in table 1. The difference between males and females in each strain was not statistically significant. There was no significant difference of liver-glucuronidase activity among the A, dba, C, and C57 black strains. The liver-glucuronidase activity of the C3H (Heston) mice was found to be considerably lower than that of the other strains, the difference being sevenfold. The livers of the C3H mice, with or without the milk factor, showed essentially similar glucuronidase activity.

TABLE 1.—*The liver-glucuronidase activity and incidence of spontaneous tumors of inbred mouse strains*

Strain	Incidence mammary tumors [1] (*10, 11*)	Incidence spontaneous hepatoma (*12, 13*) [2]		Number of Animals		Liver-glucuronidase activity [3]			
		Male	Female	Male	Female	Mean male	Mean female	Mean group	Standard deviation mean
	Percent	*Percent*	*Percent*						
C3H	91	26	6	10	10	0. 37	0. 42	0. 39	±0. 05
C3H f [4]	0–10	------	------	5	5	. 26	. 38	. 32	±. 09
dba	66	<0. 5	<0. 5	15	15	4. 1	3. 4	3. 8	±. 8
A	43	. 7	. 7	10	10	4. 0	3. 4	3. 7	±. 7
C	1	------	0	10	10	3. 8	3. 7	3. 7	±. 8
C57 black	0. 5	<0. 5	<0. 5	8	7	4. 5	3. 8	4. 2	±. 6

[1] In-breeding females.
[2] Law, Lloyd: Personal communication.
[3] Expressed in arbitrary units, see text.
[4] Foster-nursed on C57 black mothers.

DISCUSSION

Comparison of the liver-glucuronidase activity with the reported incidence of spontaneous mammary tumors in the strains employed (*10, 11*) shows no close correlation (table 1). The presence or absence of the milk factor in C3H mice did not apparently influence the liver-glucuronidase activity.

Various observers have reported an incidence of spontaneous hepatomas in C3H mice greater than that in other strains (*12, 13*).[4] Further investigations of the relationship of glucuronidase to the development of hepatomas in C3H mice have been initiated.

The low glucuronidase activity in C3H livers may be due to the presence of a glucuronidase inhibitor such as ascorbic acid or heparin (*17*). However, the presence of such a substance was not detected when mixtures of C3H and dba liver homogenates were assayed. There was reduced enzyme activity only in proportion to the dilution of the high enzyme component (dba) (table 2).

Preliminary data indicate that the low liver glucuronidase of the C3H strain may be genetically determined. Breeding experiments to confirm this are in progress.

[4] Law, Lloyd, National Cancer Institute: Personal communication.

TABLE 2.—*Effect of addition of homogenate of C3H liver to homogenate of dba liver*[1]

Tube No.	C3H homogenate	dba homogenate	C3H homogenate	Glucuronidase activity[2]
	Milliliters	*Milliliters*	*Percent*	
1	0.0	2.0	0	2.8
2	.1	1.9	5	2.8
3	.2	1.8	10	2.7
4	.5	1.5	25	2.4
5	1.0	1.0	50	1.8
6	2.0	0	100	.43

[1] 306 mg. of C3H liver and 306 mg. of dba liver were each homogenized in 10.0 ml. of distilled water. 2.0 ml. portions of these homogenates and the mixtures shown above were assayed in duplicate.
[2] Expressed in arbitrary units, see text.

SUMMARY

The livers of 115 mice of 5 inbred strains were assayed for glucuronidase activity. The liver-glucuronidase activity of C3H mice was 80–90 percent lower than that of the A, dba, C, and C57 black strains. The explanation of this difference is not apparent, although a relationship to the high incidence of hepatomas in C3H mice is suggested. There seems to be no close correlation between liver-glucuronidase activity and the reported incidence of spontaneous mammary tumors.

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PREPARATION OF *p*-DIMETHYLAMINOAZOBENZENE CONTAINING ISOTOPIC CARBON IN THE DIMETHYLAMINO GROUP [1]

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We have recently reported (*1*) the preparation of *p*-dimethylaminoazobenzene (hereafter called DAB) containing N^{15} in each of the possible positions. DAB thus prepared makes it possible to study the fate of the nitrogen-containing moieties of this hepatic carcinogen. It also affords a means of studying the possible mechanism by which DAB produces hepatic tumors. Inasmuch as *p*-aminoazobenzene has been found to be noncarcinogenic (*2, 3*), the methyl group of DAB probably plays an important role in the production of hepatomas. The preparation of DAB containing C^{13} in one of the methyl groups reported here makes available a substance which can be used in studying this possible role of the methyl group.

The first route explored, which it was hoped would lead to the desired compound, was the methylation of N-acetyl–N-methyl–*p*-aminoazobenzene (*4*) with C^{13} methyl iodide. However, all attempts to carry out this reaction with or without a buffer to take up the hydrogen iodide formed, led to cleavage of the azo-linkage.

The desired DAB was obtained by methylating methylaniline with C^{13}-methyl iodide which was followed by condensation of the resulting isotopic dimethylaniline with diazotized aniline as previously described (*1*). It was not necessary to isolate the dimethylaniline, but it was used as an aqueous solution of its hydrochloride.

Using nonisotopic material, better over all yields consistently resulted when no buffer was used during the methylation reaction. This may be contrasted with the superior yields obtained in the methylation of aniline to dimethylaniline in the presence of a sodium acetate buffer (*1*).

EXPERIMENTAL

DIMETHYLANILINE HYDROCHLORIDE

By means of a vacuum line (*5*), 4.80 gm. of methyl iodide (C^{13} atoms percent excess, 56.7)[2] were transferred to a glass pressure tube containing

[1] Received for publication October 11, 1949.

[2] C^{13} analysis as reported by Eastman Kodak.

3.62 gm. of methylaniline. The tube was sealed off and heated at 100° C. for 17 hours after which time the tube was cooled in ice and opened. The contents of the tube were made alkaline and steam distilled; the distillate was trapped in a solution of 10 ml. of concentrated hydrochloric acid in 10 ml. of water.

DAB

Following the procedure described previously (*1*), aniline, 3.15 gm., was diazotized and coupled, in the presence of sodium acetate as a buffer, with the dimethylaniline hydrochloride prepared above.

After standing overnight, the precipitated material was collected by filtration and after two recrystallizations from 95 percent ethanol there was obtained 4.9 gm. (64.5 percent, based on methyl iodide) of DAB, m. p. 114–116° (corrected).

Analysis[3] Calculated for $C_{14}H_{15}N_3$: C, 74.6; H, 6.7; found: C, 75.0, 74.7, 75.3; H, 6.5, 7.1, 6.7 (C^{13} at percent excess. Calculated: 4.1; found: 4.4, 4.4).[4]

SUMMARY

p-Dimethylaminoazobenzene containing C^{13} in the dimethylamino group has been prepared by the methylation of methylaniline with C^{13}-methyl iodide followed by coupling of the resulting dimethylaniline with diazotized aniline.

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CUTANEOUS MELANIN PRODUCTION IN MICE FOLLOWING APPLICATION OF THE CARCINOGEN 5, 9, 10-TRIMETHYL-1,2-BENZANTHRACENE [1]

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Lipschütz (*1*) in 1924 reported that papillomas and pigmented foci developed in the skin and subcutaneous tissues of mice after painting with an extract of crude coal tar. Badger and his coworkers (*2*) reported melanomas in 3 of 66 mice receiving repeated applications to the skin of a dilute solution of 5,9,10-trimethyl-1, 2-benzanthracene. Using the technique described by Badger, Hartwell and Stewart (*3*) reported the induction of local malignant tumors and focal areas of pigmentation in the skin of mice but no melanomas.

The present experiment was designed to study the pathogenesis of the focal areas of pigmentation and to attempt to induce malignant melanomas in the skin of mice by the cutaneous application of 5,9,10-trimethyl-1, 2-benzanthracene. A malignant melanoma in the skin of a guinea pig has been recently reported following application of the chemically related hydrocarbon 9,10-dimethyl-1,2-benzanthracene (*4*).

MATERIALS AND METHODS

The polycyclic hydrocarbon 5,9,10-trimethyl-1,2-benzanthracene (m. p. 127°–128° C.) has been shown to be a powerful carcinogenic agent (*2*, *3*). Twice weekly a concentration of 0.06 percent of this substance in thiophene-free benzene was painted on the dorsal skin of mice with a No. 5 camel hair brush from the level of the iliac crest to the base of the tail. Two hundred and seventy-five animals of strains dba, C57 black, and their F_1 hybrids were employed (table 1); they were approximately equally divided as to sex, were 6–8 weeks of age, and were selected from stocks maintained at this Institute. They were fed a diet of Purina animal laboratory chow with water ad libitum. At no time were they exposed to direct sunlight or to an artificial source of ultraviolet light radiation. Twenty-four control animals were painted twice weekly with thiophene-free benzene for varying lengths of time (table 2). These mice were of the same strains as those treated with the carcinogen.

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TABLE 1.—*Carcinogen-treated mice*

Strain	Number	Male	Female	Age at death (months)																		
				3	4	4.5	5	5.5	6	6.5	7	7.5	8	8.5	9	9.5	10	10.5	11	11.5	11.75	12	12.5
dba	73	41	32	0	0	1	2	2	2	2	2	4	8	6	11	7	9	8	5	2	0	2	0
C57 black	58	30	28	1	0	0	0	0	0	0	1	2	3	2	4	7	11	11	10	1	1	3	0
dba X C57 black	67	32	35	0	1	0	3	0	2	1	2	5	9	1	12	5	9	4	7	4	0	3	0
C57 black X dba	77	40	37	0	0	0	1	1	1	0	2	2	2	4	9	6	15	10	9	7	0	2	6
Total	275	143	132	1	1	1	6	3	5	3	7	13	22	13	36	25	44	33	31	14	1	10	6
Percent																							

Strain	Painting time (months)													Findings								
	1.5	2	2.5	3	3.5	4	4.5	5	5.5	6	6.5	7	7.5	Dyskeratosis	Papilloma	Carcinoma	Sarcoma	Focal Pigmentation	Carcinoma with Pigmentation	Sarcoma with Pigmentation	Carcinoma and Sarcoma with Pigmentation	Metastases
dba	0	0	2	3	1	1	1	13	2	14	4	32	0	36	39	43	9	46	25	4	3	7
C57 black	1	0	0	0	0	2	0	0	0	8	4	42	1	17	12	52	9	36	32	4	3	9
dba X C57 black	0	1	0	1	0	2	0	8	1	20	3	31	0	18	21	61	16	51	46	12	12	19
C57 black X dba	0	0	0	0	1	1	0	4	0	27	2	42	0	23	28	66	20	59	52	15	13	24
Total	1	1	2	4	2	6	1	25	3	69	13	147	1	94	100	222	54	192	155	35	31	59
Percent														34	36	80	19	70	56	12	11	21

TABLE 2.—*Benzene-treated mice*

Strain	Number	Male	Female	Painting time (months)					
				4	9	9.5	12	14	16
dba × black	6	2	4	2	3	1	0	0	0
C57 black	8	6	2	1	3	3	1	0	0
dba	10	5	5	1	3	0	3	2	1
Total	24	13	11	4	9	4	4	2	1

Mice were selected for autopsy when they exhibited small and large tumors or when moribund. Post-mortem examination was complete, and tissues taken for study included bone, bone marrow, internal viscera, central nervous system, and the painted area. Pieces of tissue were placed in the following fixatives: Zenker's acetic acid fluid, Bouin's fluid, dilute formaldehyde solution (U. S. P. 1:10 and U. S. P. 1:100).

The histochemical method utilized for the demonstration of the enzyme "dopa-oxidase" was adapted from that described by Laidlaw (*5*).

Thin pieces of fresh tissue were fixed in a dilute formaldehyde solution (U. S. P. 1:100) for ½ to 1 hour. A large number of frozen sections 15–25 micra in thickness were cut. These were immediately placed without washing in a buffered "dopa" solution (1:1,000) (pH 7.4) in petri dishes and incubated for one-half hour. They were then transferred to a fresh buffered dopa solution where they remained for 1 to 3 hours, depending on the intensity of the reaction as determined by periodic microscopic examination.

Fresh frozen sections were also placed in distilled water for 3 to 4 hours to serve as controls.

Finally the sections were washed in distilled water, dehydrated in alcohol, cleared in xylol, and mounted in balsam. Before the dehydration, clearing, and mounting procedures, some were lightly counterstained with hematoxylin, or hematoxylin and eosin, to provide cellular detail.

Tissues selected for the dopa technique were the following: dorsal skin painted with the carcinogen for periods of 3 to 7 months, dorsal skin from control animals painted with benzene for 6 months, and dorsal skin from C57 black mice 6 months of age which had received no experimental treatment.

The routine histologic stain used for all other tissues was hematoxylin and eosin.

Special stains and impregnation methods were applied to paraffin sections when considered necessary and included Wilder's silver impregnation method for reticulum, van Gieson's stain for collagen, the toluidine blue stain, the Prussian blue reaction for iron, and Krajian's silver impregnation method for melanin.

RESULTS

Hair loss was the earliest clinical manifestation of the action of the carcinogen on the painted area (Figs. 1, 2, and 3). This was followed shortly by scaliness of the superficial epidermis and hyperkeratinization.

Papilloma formation and cutaneous ulceration likewise occurred early. Multiple small pigmented foci first appeared after 3 months' application of the carcinogen. They were located in intact skin or at margins of ulcers or at sites of papilloma formation. These pigmented foci were observed in 192 of the animals, an incidence of 70 percent. They varied in diameter from less than 1 to over 5 mm. and appeared black in animals of strain C57 black and the F_1 hybrids, and brown in those of strain dba. The pigmented areas were often incorporated by the margins of the malignant tumors, following which the pigmented areas ceased to enlarge and were often broken up.

In histologic sections, the pigment gave a positive reaction with Krajian's silver impregnation method and was bleached (*6*) following the application of potassium permanganate and oxalic acid. The Prussian blue technique for iron was negative. This evidence suggested that the described pigment was actually melanin.

Microscopically, the major part of the pigment was contained within cells in the dermis, with relatively negligible amounts in epidermal cells (Figs. 12 and 13). In tissue sections bleached by the potassium permanganate method, the cells of the lesion were large, averaging 14 micra in diameter, usually polyhedral in shape, with abundant, faintly granular cytoplasm (Fig. 14). Each cell contained a single, small, uniform, slightly eccentric nucleus. The periphery of the cell was sharply outlined and well demarcated, and there was no evidence of dendritic processes (Fig. 15). These microscopic features suggested that these cells were macrophages which had ingested melanin, becoming melanophores. Pigmented lesions stained by the dopa technique did not show dopa-oxidase activity. One reason for this may have been because the cells of the lesions were so stuffed with black pigment granules that a positive reaction could not be discerned.

The pigmented foci were found in the painted area in association with atrophic, acanthotic, or neoplastic cutaneous epithelium or subcutaneous sarcomas. Occasionally, the pigmented cells appeared to be broken up and destroyed by invading carcinoma or sarcoma, with release of melanin granules (Fig. 19). Under these circumstances, the melanin granules were found between, superimposed on, or actually within the neoplastic cells. In most instances in which malignant cells contained melanin, phagocytosis seemed to be the explanation for it. Small squamous-cell carcinomas were occasionally found associated with numerous melanin granules (Fig. 11).

In one notable instance, however, the tumor cells appeared to have actually formed the melanin and on this basis, this one tumor was considered to be a melanoma (Figs. 20, 21, and 22).

Autopsy No. F-3235: Mouse, strain dba, female, 11 months of age, painted twice weekly with 5,9,10-trimethyl-1,2-benzanthracene in benzene for 221 days. Etherized.

Gross findings: Situated just above the base of the tail on the dorsal surface there is a firm rounded tumor measuring 11 by 9 by 4 mm. The

skin overlying this mass shows hyperpigmentation while the surrounding skin possesses many small pigmented areas up to 3 mm. in diameter which appear to radiate from the main tumor. The skin extending from the upper margin of the tumor is dry and scaly and gives rise to several papilliferous excrescences, the largest of which is 5 mm. in diameter. On section a small amount of dark red fluid escapes, the tumor itself being composed of firm brown-black tissue.

Microscopic findings: A large circumscribed, partially encapsulated pigmented tumor occupies the subcutaneous tissue. Tumor cells are predominantly fusiform in shape, their cytoplasm containing a multitude of fine brown pigment granules. Nuclei are hyperchromatic, oval, or spindle shaped and contain a single large nucleolus. Interspersed between the tumor cells are globular or polyhedral cells which are more deeply pigmented. These have the morphological appearances of melanophores in bleached sections. At one margin pigmented tumor cells are invading the adjacent subcutaneous tissue.

The overlying epidermis is thin and atrophic and is separated from the tumor by approximately 1 mm. of edematous connective tissue. In one area at the periphery of the tumor, epidermoid carcinoma is invading the subcutaneous tissue.

Impression: Pigmented spindle-cell tumor with local invasion (malignant melanoma).

In this one instance the pigment which was found in tumor cells could not be explained by phagocytosis. In our opinion, only cellular synthesis could have produced such a widespread pigmentation of the tumor cells. This tumor was transplanted to three dba mice, but unfortunately infection ensued and the animals died shortly.

Epidermoid tumors arising in skin varied histologically from well-differentiated types with pearl formation to anaplastic spindle-cell carcinomas. They were associated with papillomas, areas of focal hyperkeratosis, cutaneous atrophy, ulceration, inflammation, and fibrosis. They invaded subcutaneous tissue, muscle, and fascia in late stages and often penetrated blood vessels and lymphatic spaces. Metastatic carcinoma was found in 59 cases, usually in the regional lymph nodes and the lungs, and in 2 cases in the adrenal gland.

Sarcomas appeared later in the course of skin painting than did the carcinomas. Histologically most of them were fibrosarcomas, but some resembled myosarcomas while still others were undifferentiated. No intensive study of the histogenesis of these tumors was made, and they are merely tabulated as sarcomas.

Eleven mice painted with the carcinogen developed carcinoma of the mammary gland.

In mice treated with the carcinogen for periods of 5 to 7 months, skin subjected to the dopa technique exhibited numerous melanoblasts at the dermal-epidermal junction (Fig. 8). The distribution of the melanoblasts appeared random, in some areas being clumped and multiple,

while in others they were single or absent. Collections of melanoblasts were often found at the dermal-epidermal junction in close proximity to the subcutaneous melanophore aggregates. It could not be ascertained whether or not melanoblasts were present in the melanophore areas, since the extreme melanization of the area precluded the possibility of distinguishing between melanoblasts and melanophores. In bleached sections, differentiation by the dopa reaction was likewise impossible because of loss of enzyme activity.

A direct relationship was apparent between the state of the skin epithelium and the presence or absence of melanoblasts. Normal skin, skin painted with benzene, and atrophic skin showed no evidence of melanoblastic activity. On the other hand, in acanthotic and early neoplastic conditions such as carcinoma *in situ*, melanoblasts were often numerous and apparently active (Figs. 9 and 10). If, however, neoplastic alteration had proceeded further and the cancer was invading, no melanoblasts were found (Figs. 16, 17, and 18).

CONTROL SERIES

No carcinoma or sarcoma arose after painting twice weekly with thiophene-free benzene. Proliferation of hair follicles (Fig. 4, *A*) and dermatitis were constant features. No pigmented foci were demonstrable histologically. In dopa-treated sections of skin from the benzene-painted mice, one to several dendritic melanoblasts were found at the dermal-epidermal junction (Fig. 4, *B*) or in the subcutaneous tissue between the hair follicles.

Melanoblasts have been reported to be absent in the skin of normal mice except for those in hair follicles (7). Untreated mouse skin in this experiment did not contain characteristic melanoblasts (Fig. 5), but certain cells of the epidermis were pigmented by dopa in a manner to suggest that dopa-oxidase-containing cells were actually present (Fig. 6). They were usually elongated, lying within the epidermis or at the dermal-epidermal junction. No dendrites could be discerned. Single, dendritic dopa-positive cells were occasionally seen in the subcutaneous tissue (Fig. 7).

DISCUSSION

This experiment demonstrates that the carcinogen 5,9,10-trimethyl-1,2-benzanthracene is capable of inducing carcinomas, sarcomas, melanoblastic proliferation, and melanophore aggregates. The evocation of carcinoma and sarcoma indicates that in this respect the carcinogen does not differ from others, such as 20-methylcholanthrene, although the latent period of tumor induction may be somewhat longer. Pigment formation in mice following the application of this carcinogen to the skin is a singular result not often encountered with other carcinogens.

The actual mechanism of melanin formation is unknown. "1-Dopa," or levorotatory 3,4-dihydroxyphenylalanine, has been suggested as the intermediary substance in the biological formation of melanin from the

amino acid l-tyrosine, but it has not as yet been isolated from tissues. The enzyme "dopa-oxidase" as contained in melanoblasts is considered to be a prerequisite for the formation of melanin. This is borne out in histologic studies, as tissues possessing large amounts of this enzyme are usually deeply pigmented, for example, melanomas (*8*). The skin of albino animals, areas of vitiligo, and fresh skin scars, all amelanotic, are devoid of melanoblasts as determined by the dopa technique.

The basis of the dopa reaction is simply the conversion by oxidation of dopa to a colored polymer which is precipitated at the site of oxidase activity. A melanoblast would not react with dopa if it contained no enzyme. Conversely, a high content of enzyme would be revealed by a heavy precipitation of dopa. Intermediate concentrations of the enzyme would give corresponding reactions. This method allows a rough approximation of enzyme activity of melanoblasts, provided the staining time remains a constant.

A further criterion of the activity of the normal melanoblast is presence of enzyme in thin, tenuous ramifications of the cell cytoplasm. These processes are distributed between but in close contact with cells of the epidermis. When the melanoblast is in the inactive or dormant state, enzyme is absent or present only in the main cell body. Complete activity is characterized by the presence of enzyme throughout the cell including all of its processes. The dopa reaction can thus reveal the functional activity of melanoblasts by these two characteristics, first the relative intensity of the reaction, and second, the distribution of the enzyme in these cells.

Increased melanin formation may occur through the production of excessive amounts of dopa oxidase by the normal melanoblastic components of skin. Again, a multiplication of these cells *in situ* would provide extra amounts of the enzyme. Thirdly, melanoblasts may migrate from other locations (*9*) to a particular area and, by their aggregation, produce a high local concentration of the enzyme.

In this experiment carcinogen-treated mouse skin has been shown to contain increased numbers of melanoblasts which possessed increased functional activity, as indicated by intensity of dopa reaction as well as enzyme-filled processes. Their increase in numbers may have been due to local multiplication, or migration from other sources such as hair follicles. Subcutaneous melanin deposits in melanophores characterized the late effect of chronic stimulation of melanoblasts (Fig. 12).

The relationship between the cutaneous changes induced by painting and the presence of melanoblasts merits comment. Normally, the epithelium of the skin of the mouse is from two to four cell layers thick. After painting with the carcinogen, thickening of all cell layers becomes evident. It is in the stages of hyperplasia and neoplasia *in situ*, in which the epithelium of the skin may consist of nine or more layers of cells, that melanoblasts are most prominent and abundant.

Thus, it may be postulated that the action of the carcinogen is exerted not only upon the skin epithelium but upon the melanoblastic components as well.

Concomitant hyperplasia of both elements ensues until or shortly after carcinoma has been induced. Then possibly the carcinogen becomes too toxic for the melanoblasts, or the proliferative rate of the melanoblasts does not match that of the skin epithelium and so they are encompassed by advancing tumor cells.

One pigmented tumor induced in the course of this experiment had the morphological appearance of a melanoma and is tentatively considered to be such.

SUMMARY AND CONCLUSIONS

The carcinogen 5,9,10-trimethyl-1,2-benzanthracene is capable of inducing skin carcinoma, subcutaneous sarcoma, and melanotic foci in mice. The melanotic foci have been shown to consist largely of macrophages which are acting as melanophores. One pigmented tumor has been described as a malignant melanoma.

A proliferation of melanoblasts has been demonstrated in the skin of mice treated with the carcinogen. The relationship of the melanoblasts to pigmented foci and to the epithelial components of the skin has been discussed.

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Plates

PLATE 92

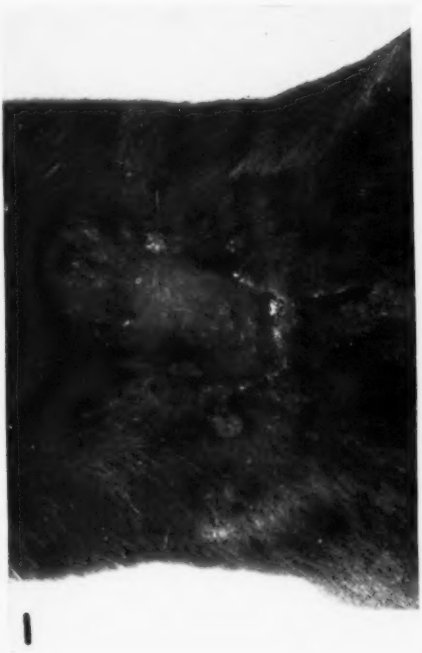
FIGURE 1.—C57 black mouse painted with carcinogen for 5½ months, showing cutaneous ulceration with marginal focal pigmentation and papilloma formation. × 3.

FIGURE 2.—F_1 hybrid of C57 black × dba painted with carcinogen for 6 months, showing ulceration with focal pigmentation and hair loss in surrounding skin. × 3.

FIGURE 3.—C57 black mouse painted with carcinogen for 5 months, showing ulceration, marginal pigmentation, and alopecia. × 3.

FIGURE 4, *A*.—Control mouse, strain dba, painted with thiophene-free benzene for 6½ months, showing proliferation of hair follicles in the painted area which has been clipped. Natural size.

FIGURE 4, *B*.—A melanoblast possessing two coarse dendrites is situated at the dermal-epidermal junction in a section of skin from a control mouse, strain C57 black, painted with thiophene-free benzene for 6½ months. Frozen section. Dopa reaction. × 1,100.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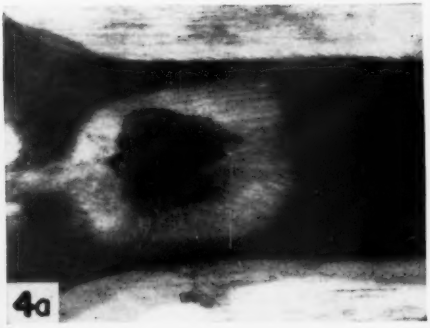
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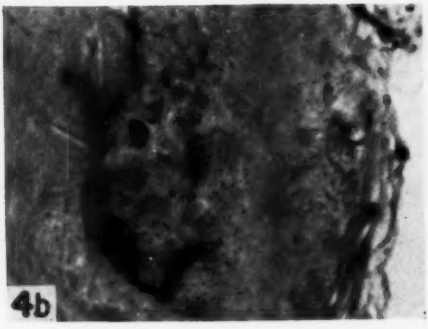
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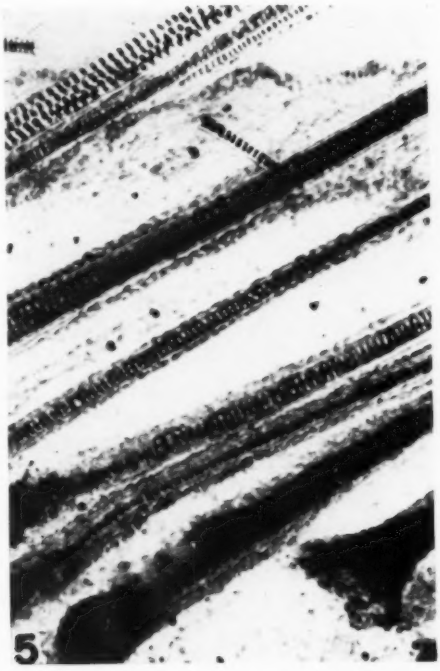
PLATE 93

FIGURE 5.—Section of skin from untreated C57 black mouse 6½ months of age. Note relative thinness of epidermis at top of figure and absence of melanoblasts. Polymorphonuclear leukocytes in the corium show a positive dopa reaction. Frozen section. Dopa reaction. × 125.

FIGURE 6.—Three cells in the epidermis of an untreated mouse show a reaction with dopa. These are probably melanoblasts, but they lack the characteristic enzyme-filled processes. Frozen section. Dopa reaction. × 1,300.

FIGURE 7.—Dendritic melanoblasts found deep in the corium of an untreated dba mouse. Frozen section. Dopa reaction. × 1,100.

FIGURE 8.—Proliferation of melanoblasts at the dermal-epidermal junction in a section of skin from an F_1 hybrid mouse. Melanoblasts have high content of enzyme, with extensive dendritic processes. Frozen section. Dopa reaction. × 385.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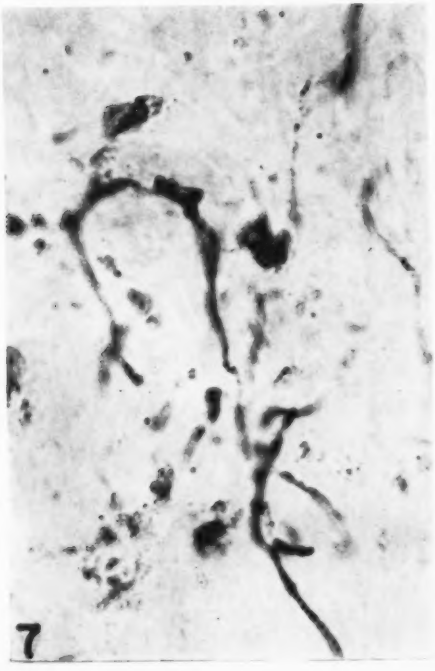
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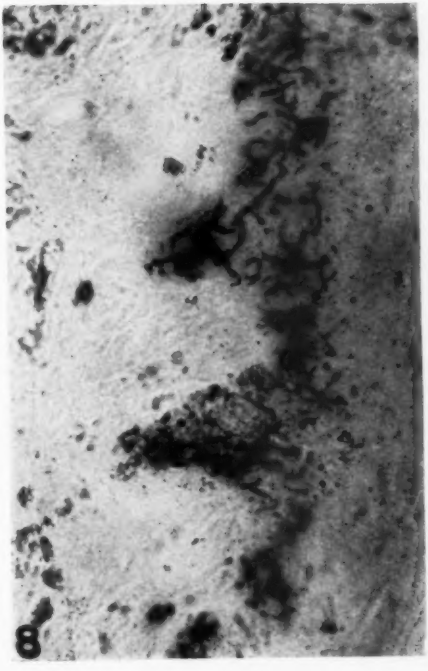
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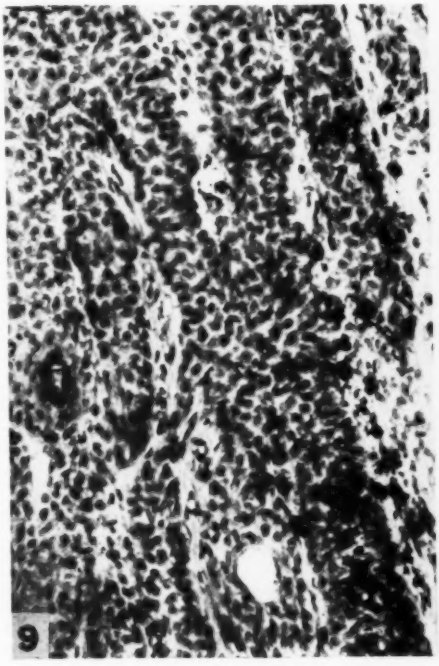
PLATE 94

FIGURE 9.—Portion of acanthotic epidermis with hyperplastic rete pegs after 5 months painting with the carcinogen. Proliferated melanoblasts are evident in association with the rete pegs. Frozen section. Dopa reaction. Hematoxylin counterstain. × 200.

FIGURE 10.—Acanthotic epidermis showing spiderlike appearance of melanoblasts. Frozen section. Dopa reaction. Hematoxylin counterstain. × 385.

FIGURE 11.—Small squamous-cell carcinoma arising from epidermis of F_1 hybrid mouse showing extensive pigmentation of tumor cells. Pigmented melanophore focus to the right. Paraffin section. Hematoxylin and eosin stain. × 86.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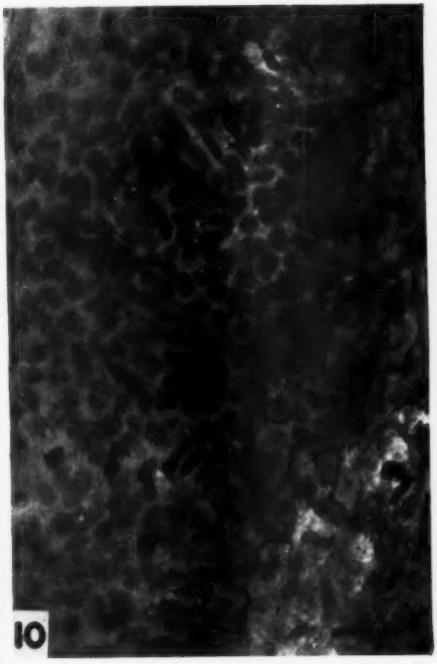
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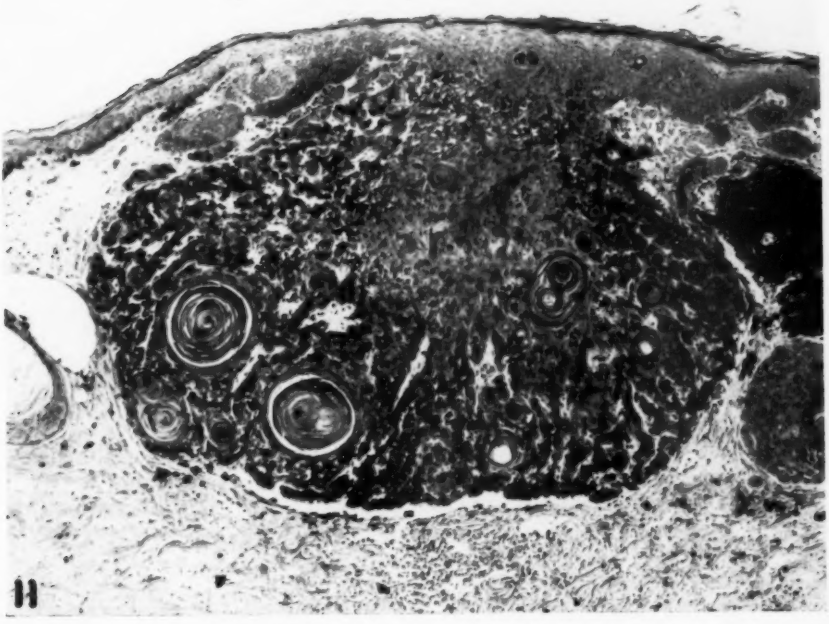
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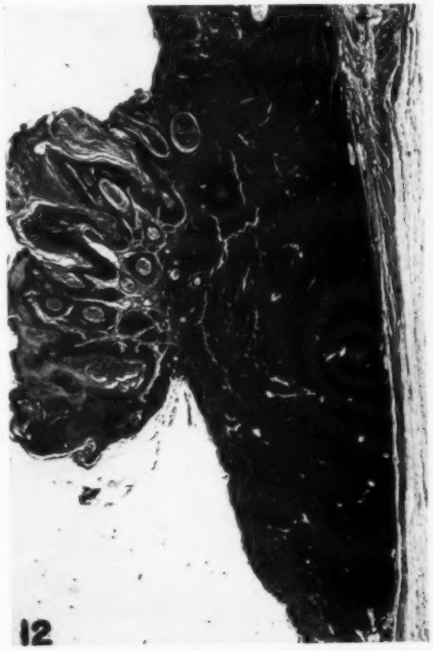
PLATE 95

FIGURE 12.—Keratinizing papilloma in F_1 hybrid mouse with extensive melanin deposit in corium. Paraffin section. Hematoxylin and eosin stain. × 20.

FIGURE 13.—Higher power view of figure 12 to show globular shape of pigment-containing cells. Paraffin section. Hematoxylin and eosin stain. × 250.

FIGURE 14.—Serial section of pigmented nodule shown in figure 12 bleached to reveal cellular detail. Paraffin section bleached with potassium permanganate. Hematoxylin and eosin stain. × 250.

FIGURE 15.—Detail of figure 14. Nuclei are small and dark, eccentric in position. Cytoplasm is abundant, with no evidence of dendritic processes. These cells considered to be melanophores are stuffed with faint-staining melanin granules. × 1,040.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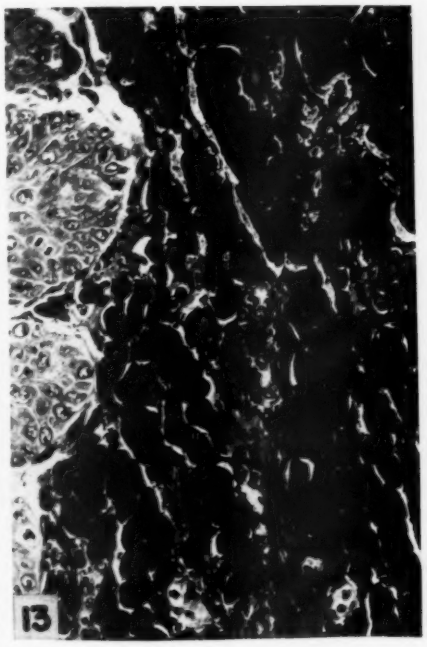
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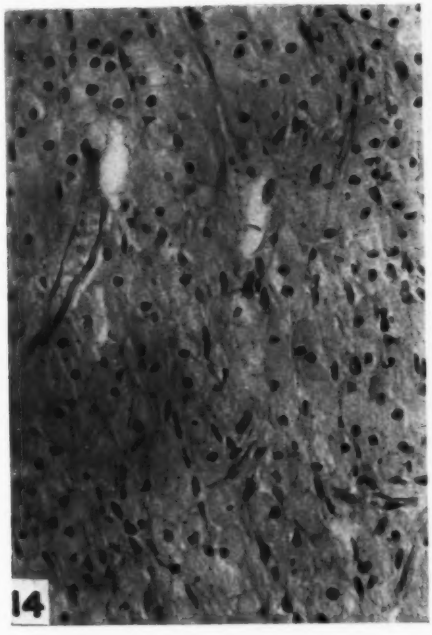
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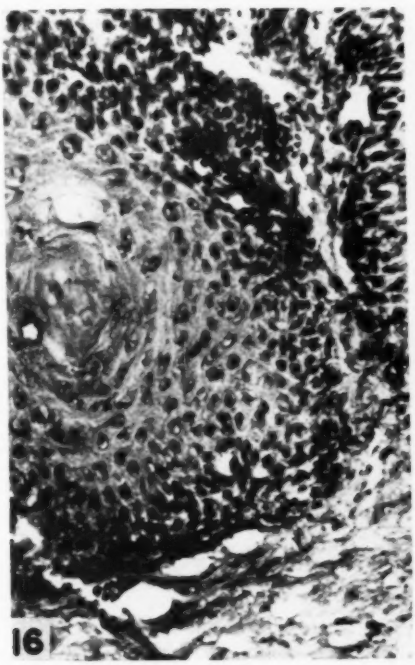
PLATE 96

FIGURE 16.—Periphery of a squamous-cell carcinoma showing dopa positivity of certain marginal cells. None possess dendritic processes, indicating declining activity of melanoblasts. Frozen section. Dopa reaction with hematoxylin counterstain. × 200.

FIGURE 17.—Squamous-cell carcinoma with occasional peripheral cells reacting positively with dopa. No dendritic processes. Frozen section. Dopa reaction with hematoxylin counterstain. × 340.

FIGURE 18.—Advancing margin of squamous-cell carcinoma containing two deeply pigmented polyhedral cells. These may be either melanoblasts or melanophores. Frozen section. Dopa reaction, hematoxylin counterstain. × 340.

FIGURE 19.—Spindle-cell carcinoma invading an aggregation of melanophores, showing phagocytosis of melanin granules by the tumor cells. Paraffin section. Hematoxylin and eosin stain. × 1,300.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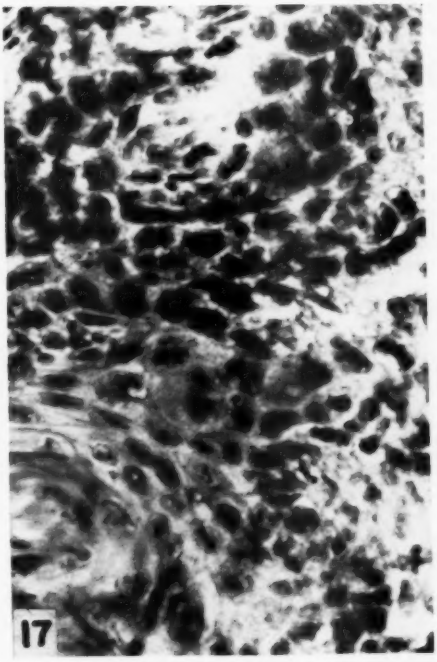
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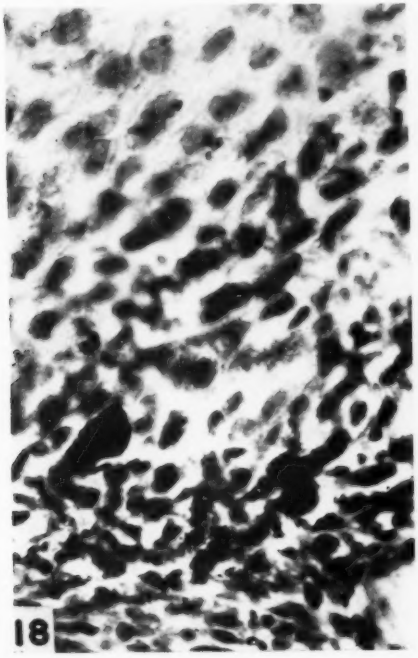
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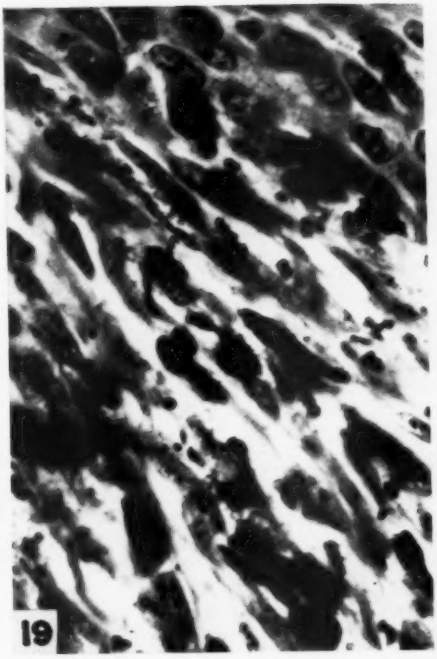
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PLATE 97

FIGURE 20.—Pigmented tumor arising in corium after 221 days' painting with the carcinogen. Compression of surrounding stroma with infiltration of one area indicates an expanding, invasive growth. The tumor is densely pigmented, consistent with the characteristics of a melanoma. Paraffin section. Hematoxylin and eosin stain. × 20.

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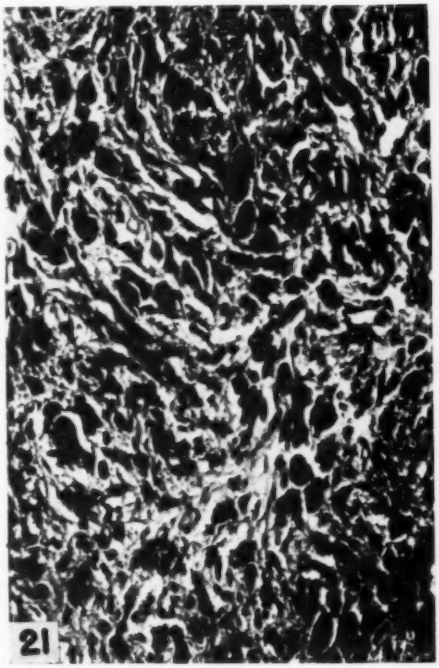
PLATE 98

FIGURE 21.—The central portion of the pigmented tumor shown in figure 20. Many globular, polyhedral cells of the melanophore type can be distinguished, surrounded by deeply pigmented tumor cells. Paraffin section. Hematoxylin and eosin stain. × 310.

FIGURE 22.—Peripheral part of the tumor shown in figure 20, with invasion of surrounding fibrous tissue by pigmented tumor cells. Paraffin section. Hematoxylin and eosin stain. × 96.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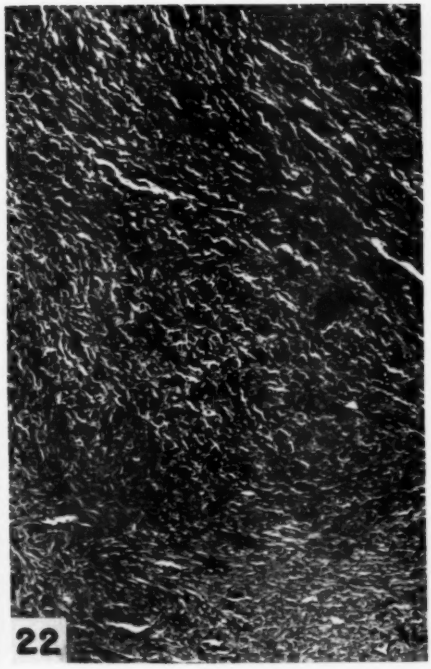
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EFFECTS OF PODOPHYLLIN ON MOUSE SKIN. III. A STUDY OF EPIDERMAL FIBRILS[1,2,3]

LESTER S. KING, M. D.

The fibrillar components of epidermis and their relation to keratinization have received virtually no attention in this country. The early observations of Schulze and Bizzozero as quoted by Shapiro (*1*), Ranvier (*2*), and Herxheimer (*3*), between 1864 and 1889, defined the intercellular bridges and the intraepidermal fibrils. Since then a very voluminous European literature has arisen, the earlier portion of which was well covered by Weidenreich (*4*) in 1900. In 1926 Patzelt (*5*) painstakingly analyzed the literature to that date. The detailed anatomical studies, presenting the morphology of fibrils in various animal species, need not be further reviewed.

In recent years the functional importance of the epidermal fibrils and their relation to keratinization has received sporadic attention, with the use of newer physical and histochemical techniques. The basis for such analysis was laid by Unna and Golodetz (*6*) in 1907, who, by means of digestion studies, distinguished three different types of keratin, of which only the first two are relevant. These authors distinguished keratin A, found in the membrane of cells of the horny layer, resistant to enzyme and acid digestion; and keratin B, found in the cell contents of the horny layer, less resistant to digestion. A totally different mode of approach was offered by Astbury (*7*), using X-ray diffraction studies on keratinized material. A characteristic X-ray diffraction spectrum was found to be substantially the same in all forms of mammalian keratin. This initial spectrum was the distinguishing sign of so-called keratin α. The same keratinized material, when stretched in water, yielded a different type of diffraction pattern, designated as keratin β. It was maintained that keratin α and β were stereoisomers, with a transformation effected by regulation of tension. The identification of keratin by X-ray diffraction is thus rendered more secure by the test of transformation from keratin α to keratin β.

The use of polarized light in the study of keratin and keratinization

[1] Received for publication October 4, 1949.
[2] From the laboratory of the Illinois Masonic Hospital, Chicago, Ill.
[3] Aided by a Grant from the National Institutes of Health.

received special impetus from the works of Schmidt (*8*, *9*) and more recently of Jaeger (*10*). The birefringence of tonofibrils forms an important buttress for interpretations of the function of these structures. Derkson and coworkers (*11*, *12*) and Champetier and Litvac (*13*) have presented evidence that the doubly refractile tonofibrils are responsible for the specific X-ray diffraction pattern of keratin α.

Derkson and Heringa (*11*) utilized a simple but ingenious technique. Studying the thick epidermis of the upper lip of the cow, they cut horizontal frozen sections at 100 microns, parallel to the surface. Sections from different levels were subjected to X-ray diffraction analysis. The keratinized portions from the surface, and the nonkeratinized deeper mucosal layers both yielded identical diffraction spectra, characteristic of keratin α. Both also showed transformation to keratin β on stretching. Thus X-ray showed an identical micellar structure in both keratin and the deeper epidermis. Derkson, Heringa, and Weidinger amplified these observations, concluding that the doubly refractile tonofibrils, common to both keratin and underlying epidermal cells, constitute the microscopically perceptible form of keratin. Champetier and Litvac, in subsequent detailed studies, conclude with Heringa and Derkson that the epidermal fibrils are responsible for the diffraction pattern characteristics of keratin α. They also claimed, by suitable digestion studies coupled with X-ray analysis, that the keratin α is associated with the keratin B of Unna, while the resistant keratin A of Unna, in the so-called cell membranes, does not exhibit the characteristic spectrum of keratin α, nor does it show birefringence.

It is unfortunate that the letters A and B, α and β, should all be coupled with the one term keratin with such very different significance. But the usage is deeply ingrained in the literature.

In view of the data presented above, morphological analysis of tonofibrillar structure is important, not only as a cytological problem, but also as a groundwork for the process of keratinization. Experimental work on the epidermal fibrils is very scanty. Since the use of podophyllin produces profound epidermal changes, the status of tonofibrils under such conditions appeared worth detailed study.

The animals and general procedures attending the use of podophyllin (or podophyllotoxin) are similar to the studies already reported (*14*, *15*). For the demonstration of fibrils the phosphotungstic-acid-hematoxylin stain, Peers modification, was employed, on formalin fixed material (*16*). This, in our hands, gave better results than primary fixation in Zenker's fluid. Routine hematoxylin-eosin stains were also utilized and in addition, in many cases, the periodic-acid-leuko-fuchsin stain (*17*), slightly modified, was used. Mice were studied at varying intervals following painting with the drug, and stages previously described (*14*, *15*) were investigated. In addition, hyperplasia of the epidermis, or acanthosis, was produced by the application of methylcholanthrene, and the structure of tonofibrils was investigated with and without subsequent application

of podophyllotoxin. Carcinomas produced by methylcholanthrene were also studied.

GENERAL CONSIDERATIONS

The term tonofibril, here used as synonymous with epidermal fibril, may be used to designate any one of three distinguishable elements. The first is the so-called intercellular bridges, which are best known in the prickle-cell layer of the human epidermis, traversing the intercellular spaces, and joining adjacent cells. Within the cytoplasmic confines there are other fibrils, which are of two types. One is the so-called spiral of Herxheimer, a coarse fibril, usually wavy and twisted, situated at the extreme periphery of the cell, and most clearly seen, under normal circumstances, in the basal layer, perpendicular to the underlying corium. The other type, constituting our third category, comprises the finer intracellular fibrils seen in the interior of the cells, especially in the prickle-cell layer.

These latter types, both intracellular, are apparently qualitatively similar. Discussion in the literature has considered the problem whether the spiral of Herxheimer constitutes the actual cell membrane. Weidenreich (*4*), and more recently Shapiro (*1*), have considered the problem and have adduced satisfactory evidence that the heavy peripheral fibril is not the cell membrane. This problem will be discussed further since it is of importance in evaluating the effects of podophyllin.

The relationship between the heavy peripheral fibril in the basal layer and the delicate more central fibers is not entirely clear. In human epidermis, for example, the Herxheimer fibers are restricted to the lowermost portions of epidermis. As the cells gradually rise towards the surface the cell orientation changes, the direction of fibers alters from vertical to horizontal, and the fibrils appear delicate, numerous, and diffuse, rather than sparse, heavy, and peripheral. The study of Thompson (*18*) on the skin of various animal species indicates clearly the great variation in prominence of the coarse and fine fibrils among different species.

The relation of the intercellular bridges to the intracellular fibrils is by no means a settled problem. Shapiro presents formidable evidence that the bridges represent independent formations, not continuous with, or connected with the intracellular fibers. He reviews the older literature, which for the most part favors the opposite view. He stresses the point that the bridges are readily observable with any cytoplasmic stain, while the other tonofibrils are demonstrable only with special stains and frequently only with considerable difficulty.

The three elements distinguished above vary greatly in their ease of demonstration. The most commonly used stains are methyl violet, or some similar aniline dye, iron hematoxylin, and phosphotungstic-acid-hematoxylin. Our own experience is limited to the last named but corresponds to the data and illustrations presented in the literature, namely, that the Herxheimer fibers are most readily exhibited. The staining of

fine intracellular fibers and of intercellular bridges shows variation depending on the animal species, the position of the cell within the epidermis, and the physiological state of the cell. These factors are mentioned here to introduce a new concept—that tonofibrils exhibit a relative and variable chromophilia, dependent on physiological and functional states. Analogy may be made with previous work on silver stains (*19*), in which a concept of variable argyrophilia was enunciated. Different tissue structures such as nuclear chromatin, Alzheimer strands, glial fibrils, axis cylinders, and the like, can be graded in relation to their affinity for silver. Similarly, different types of tonofibrils can be compared in regard to ease of staining, a property which may be designated as chromophilia.

The problem arises concerning the specificity of stains and the histochemical conclusions which might be drawn from their use. It is clear that none of the dyes commonly used to demonstrate tonofibrils are in the slightest degree specific for the epidermal fibrils. Such dyes, with suitable modifications, can be used to stain various fibrillar structures of variable composition ranging from neuroglial fibrils to mitochondria. The interpretations that are derived from the use of phosphotungstic-acid-hematoxylin are based on morphological evidence, not histochemical data. The use of the periodic-acid-leuko-fuchsin stain (*17*) confers a greater degree of histochemical specificity, but its applicability to the present problem is limited.

Normal Mouse Skin

The normal mouse epidermis possesses only two layers of cells surmounted by a very narrow band of keratin. When sections are stained with phosphotungstic-acid-hematoxylin (hereafter referred to as P. T. A.) only extremely sparse tonofibrils are demonstrable. Occasional spiral fibers (the spirals of Herxheimer) can be observed in the basal layer of cells. No intercellular bridges were seen in normal mouse epidermis, with the techniques employed. In the flattened superficial cell layer small amounts of keratohyaline granules are stained with P. T. A. The overlying keratin discloses a fine line of blue-staining material, essentially homogeneous but not appearing fibrillar in character. This in turn is surmounted by wisps and shreds of material staining light tan with P. T. A.

The total tonofibrillar mass demonstrable in normal mouse epidermis is so small that adequate photographic reproduction is not possible.

Effect of Podophyllin

When normal mouse skin is treated with podophyllin or its derivative podophyllotoxin, extensive changes are produced which, as studied in hematoxylin-eosin preparations, have already been described (*14*, *15*). For ease of description, the changes have been divided into stages, dependent on histologic appearance rather than elapsed time following application.

The early stage with its enlargement of nuclei, swelling and pallor of cytoplasm with perinuclear vacuolation, and scattered mitotic distortions shows characteristic changes when examined after P. T. A. stain. The enlarged cells, with a degree of enlargement which at this stage is only moderate, almost all show a sharply defined blue-staining band or fibril, tortuous and wavy, situated at the extreme periphery of the cytoplasm. The fibril virtually surrounds the entire cell, and is most distinctly observed in the basal cells (Fig. 1, *A*). The intercellular spaces are widened, but stainable intercellular bridges are seen only with the utmost rarity. The sharp peripheral fibril surrounds not only the cells with intact nuclei but also those with mitotic activity. In the upper layers, the peripheral fibers are less distinctly visible. Usually, instead of appearing as a solid line of tortuous contour, the peripheral fibrils in the superficial layers appear as a stippled or discontinuous line. This character is discernible only with fine focusing and is not photographically reproducible.

Fibrils within the cytoplasm (as opposed to those at the extreme periphery of the cell) are extremely scarce at this stage but occasionally are demonstrable as very delicate, pale blue lines, more or less wavy, with no definite orientation. It is only in the later stages that such fibrils are present in substantial numbers.

The most superficial epidermal cells are flattened and contain keratohyaline granules in small numbers. Under the fresh influence of podophyllin, the granules are fine and powdery, only occasionally coarse and large. The flattened cells rarely show any demonstrable tonofibrils. This peculiarity of distribution of the fibrils, best demonstrable in the lowermost layers, least demonstrable in the upper layer, poses a serious theoretical difficulty. The uppermost cells are the closest to the keratin and represent the stage immediately prior to full keratinization. Yet at this stage the fibrils, allegedly the precursor of keratin, are the least developed. On the other hand, in the basal layer, farthest removed from keratinization, the fibrils are most distinct. This theoretical difficulty will be discussed subsequently.

The actual keratinized surface is narrow and irregular. There are lines, laminae and bars of heavily blue-staining material, intermingled with tan-staining components. The blue-staining substance in part resembles squames or mummified cells. Occasionally the blue homogeneity is interrupted by stipples, but a delicate tonofibrillar structure is not demonstrable in the keratinized material, in contrast to the keratinization found in squamous carcinoma, as described below.

In the florid stage, or fully developed acute podophyllin effect, occurring on the average 48 hours after the initial painting, the changes described above are accentuated. The epidermis is broadened still more; the cells are increased not only in number but in size. The peripheral fibers are clear and distinct. Occasionally as shown in figure 1, *C*, the Herxheimer fibril actually penetrates into the corium. This peculiarity of the basal

fibrils is well recognized in the literature and can be seen fairly frequently in human hypertrophied epidermis, as in the sole or palm, or in verrucae, but in the mouse material it is rare.

Accompanying the increased mass of the epidermal cells is a greatly increased quantity of intracellular (not peripheral) tonofibrils. These are clear and distinct in tissue preparations, although somewhat less distinct in the photographic reproductions (Figs. 1, *C* and *D*). The fine fibrils tend to form a tangled skein, rather than exhibiting a distinct orientation. In the superficial cells, the tonofibrils are invariably more scanty than in the deeper layers.

Intercellular bridges can sometimes be observed, represented by extremely delicate filaments that appear to cross between adjacent cells. Such bridges constitute a very minor and insignificant part of the total tonofibrillar mass. Their extreme delicacy and faint staining must be stressed, representing a minimum degree of chromophilia, and displaying a marked contrast to the bridges seen in carcinoma (Fig. 4, *A* and *B*).

In hematoxylin and eosin preparations the large swollen cells appear surrounded and limited by a membranous structure which stains rather deeply with eosin and which in earlier papers was described as a "cell membrane." The P. T. A. stain colors the so-called membrane a deep blue. Morphologic study shows its identity with the peripheral tonofibril, or Herxheimer spiral. However, whereas the true Herxheimer fibril as hitherto described in the literature is restricted to the basal layer, the membrane-like structure appearing under the influence of podophyllin, is found in cells throughout the entire epidermis. In fact, it tends to be more prominent in the upper layers of epidermis than in the lower. The Hotchkiss stain, with periodic-acid-leuko-fuchsin, supposedly quite specific for carbohydrate, stains the membrane a medium red. Frequently this component is the only portion of the cell stained by this method.

Observations on other forms of keratinization, as for example in the human heel, indicate that the "cell membranes" of Unna, the so-called keratin A, also stains vividly with the Hotchkiss technique. Digestion studies on such material, combined with P. T. A. and Hotchkiss stains, are as yet incomplete. But the available observations, combined with those in the literature, suggest that the keratinized cell membranes, or keratin A of Unna, seen normally only in the completely keratinized portion of the epidermis, are similar to the "membranes" described as part of the podophyllin effect. With this drug the membranous appearance is observed in the viable cells of the deeper epidermis, in contrast to the human sole or heel where it is seen only in fully developed keratin.

In later stages of podophyllin effect, wherein reparative changes are evident, there are corresponding changes in the tonofibrils. The total mass of tonofibrils becomes considerably increased, and the individual fibers become more distinct. As the cells become more basophilic (to hematoxylin and eosin) and transform into the hyperplastic state desig-

nated as acanthosis, the cells assume a different shape. The basal cells tend to elongate, and the picture begins to resemble the acanthosis described below, produced by methylcholanthrene. The tonofibrils take on a totally different pattern, the intercellular bridges become distinct and prominent, and the fully repaired state resembles Figure 2, *B*, produced by methylcholanthrene. However, in the process of repair there is some lag between different portions of the epidermis. Transitional forms between swollen cells and those of polyhedral shape are readily visible.

As the epidermis advances in the reparative stage, the strong peripheral tonofibrils lose their relative prominence. Correlative with this is an increase in prominence of the centrally located fibrils, and also of the intercellular bridges. There is thus an alteration in the chromophilia of the various tonofibrillar components.

A feature of extreme interest is the behavior of epidermal fibrils in the cells with severe mitotic disturbance, the so-called podophyllin cells. In the early stages such cells reveal break-up of chromatin material in characteristic fashion, while the cytoplasm shows a bubbly type of vacuolation. In the early stages the cytoplasm is bordered by a strong peripheral fibril, staining deeply with P. T. A., while the central portions are devoid of any stainable fibrils. Figure 1, *A*, shows such a cell (marked by arrow) in which the peripheral fibril is sharp, while the central part of the cell reveals three large clumps of chromatin.

By the third or fourth day, however, these podophyllin cells have increased greatly in size. The peripheral fibril is still prominent but frequently appears reduplicated in concentric fashion, with a clear space in between. Of greater significance is the development within the cytoplasm of a deep-blue staining meshwork. The strands of stainable material may be extremely thick and prominent and show a definite increment with the passage of time. For comparison with Figure 1, *A*, at 24 hours, Figure 1, *B*, at 72 hours and Figure 2, *A*, at 96 hours may be examined. Although the chromatin material and the fibrillar network appear both black in the photographs, they are clearly distinguishable in the tissue preparations.

In Figure 2, *A*, a cell with four micronuclei is visible. In cells of this type, there is a development of tonofibrils identical with those in the podophyllin cell. Since the micronuclei arise from the chromatin fragments of the podophyllin cell, such a parallelism is not surprising.

The fibrils are a product of cytoplasmic activity. The role of the nucleus in such activity is not clearly understood. There is unequivocal evidence from these studies, however, that the cytoplasmic activity concerned with fibril production continues unabated while the nucleus remains in a state of suspended mitosis. The intact nucleus is clearly unnecessary for such cytoplasmic activity. Whether individual and discrete chromatin particles can exert an effect on the cytoplasm is not known.

Strong tonofibrillar development in the cytoplasm can also take place in the presence of micronuclei. The functional relationship of micronuclei to intact nuclei is not known.

In Figures 1, *B*, and 2, *A*, the sturdy tonofibrillar development in other cells of the epidermis can be seen 3 and 4 days after application of the drug. As the central fibrils become prominent, the peripheral fibril of Herxheimer becomes less conspicuous. Other portions of such preparations show a tonofibrillar architecture comparable to Figure 2, *B*, and need not be described separately.

Cells which, in hematoxylin and eosin preparations, can be designated as degenerative can still display a considerable mass of tonofibrils of very delicate type. From the P. T. A. stains alone it is not really possible to distinguish a degenerative phase separate from the large swollen dedifferentiated type, and such distinction must be made on the basis of hematoxylin and eosin stains. However, when cells attain an extreme degree of change designated as degenerative, tonofibrils stain very poorly or not at all.

Effect of Methylcholanthrene

Two to four applications of methylcholanthrene (hereafter referred to as M. C. A.) induce a pronounced hyperplasia of the epidermis, described as acanthosis. Mitotic distortions and arrests and cytoplasmic swelling and pallor, of the type caused by podophyllin, are not produced. In the course of acanthosis following M. C. A., the tonofibrils undergo marked changes. The basal cells are elongated, cells of the middle layer are polyhedral, while those of the upper layer are flattened. There is a sharp increase in tonofibrils of all three types. Herxheimer fibers of peripheral location can be readily seen, especially in the basal cells, but do not assume the prominence seen after podophyllin. The fine intracellular fibrils vary in number, but in general are numerous and distinct. The intercellular bridges are perhaps the most striking and are observed with great clarity in the basal and middle layers, less distinctly in the superficial portions. In the granular layer, keratohyaline granules are numerous, while fibrils are scanty, inconspicuous, and frequently indistinguishable or absent. The layers of keratin present, in part, masses of purplish homogeneous material, marked off into suggestions of cell outlines. In part the keratinized layer reveals abundant fine blue dots and short lines, reminiscent of tonofibrillar fragments. Figure 2, *B*, illustrates some of the changes described.

Methylcholanthrene and Podophyllin

The initial experiments on tonofibrils began with normal mice, in whose epidermis the fibrils are very scanty and poorly developed. Under the influence of podophyllin, there occurred an extreme proliferation of tonofibrils, in spite of the severe cytological changes. The question then arose, given a mouse skin with hyperplasia of tonofibrils already estab-

lished, as after treatment with M. C. A., what effect would be produced by subsequent treatment with podophyllin?

To study this problem mice were given three applications of 0.3-percent M. C. A. in acetone, within 1 week. Four days later, to one-half of the painted area, 20-percent podophyllin ointment was applied. Mice were killed at intervals, the most instructive being 24 and 48 hours after the podophyllin. In the same preparation it was possible to observe pure acanthotic epidermis and acute podophyllin change in different portions.

The results were not so striking as might be expected. The control acanthotic skin produced by M. C. A. revealed very abundant tonofibrils, generally straight, clearly defined, with numerous distinct intercellular bridges, as described above. Under effect of podophyllin the tonofibrils presented no significant alteration in mass. However, as the cells swelled and became rounded, the intracellular fibrils pursued a wavy and tortuous course and appeared somewhat more delicate than in the control portions. The intercellular bridges for the most part remained. Except for the prominence of these bridges, the tonofibrillar picture of 24 hours of podophyllin effect superimposed upon acanthotic skin, resembled the 48- to 72-hour stage of podophyllin on normal skin. Under the effect of podophyllin, epidermis can develop a total tonofibrillar mass comparable to that produced by M. C. A., in spite of the great differences in cytoplasmic behavior under the two drugs and the differences in distribution of the individual fibrils.

The conclusion may be drawn that while podophyllin exerts its characteristic effect upon acanthotic skin, as well as normal skin, the tonofibrils are in no way diminished or destroyed. Their course, distribution, and orientation, however, do change. Figures 2, *B*, and 3, *A*, and *B*, show representative fields of M. C. A.-treated animals, with and without superimposed podophyllin effect.

Carcinoma Produced by Methylcholanthrene

The carcinomas produced by M. C. A. may vary considerably, depending upon the degree of epithelial differentiation, and extent of keratinization. Tonofibrillar studies upon such animals give the best results in well differentiated tumors showing abundant keratinization. The less differentiated tumors show very scanty and poorly defined fibrils.

The malignant cells show a remarkable development of tonofibrils but with considerable variation. The most uniformly prominent component is the intercellular bridge. Such bridges are observed throughout masses of epithelium, coursing between adjacent cells. For the most part, the tumor cells are relatively small, and intracellular fibrils are frequently inconspicuous. Sometimes only the periphery of the cell exhibits a delicate and fine fibril, comparable to the Herxheimer spiral described in normal epithelium. Even in the well-differentiated tumors, numerous cells can be observed wherein the cell outline and contents appear light tan with the P. T. A. stain, but the intercellular bridges are clear,

distinct, and sharply stained with blue (Fig. 4, *A*, and *B*). In the less differentiated tumors such appearances are the rule rather than the exception. For the most part, the well-differentiated tumors reveal very abundant fibrils within the cell confines, as well as distinct bridges.

Other cells are larger, more bizarre in shape, and may show a rich and dense fibrillar constitution. Occasionally these cells are so large and so crammed with fibrils that they resemble neurones containing neurofibrils as demonstrated by silver stain (Fig. 4, *B*).

The keratinized material produced by the squamous carcinomas shows two definite types of reaction. There are solidly keratinized plugs, and masses which stain an intense and essentially homogeneous deep blue, with the exception that cell outlines, are occasionally faintly visible. There are other zones of keratinization, especially in the epithelial pearls, where the keratinizing substance presents an intensely fibrillar constitution, with the sharp blue fibrils apparently imbedded within a light tan-staining background (Fig. 4, *C*). These latter appearances suggest an incomplete type of keratinization, with far more intense fibrillar differentiation than is ever observed in mature ripe keratin. The appearances suggest that under certain circumstances, as, for example, when keratin is being rapidly formed, the tonofibrillar components become intensely hyperplastic, producing a definite visible dense feltwork. On the other hand, other types of keratin, apparently completely mature, stained uniformly, with no trace of fibrillar differentiation persisting, with the techniques employed.

DISCUSSION

We have considered tonofibrils under 3 categories, the intercellular bridges, the peripheral cytoplasmic or intracellular fibril, and the central fibrils. It is not possible to settle the problem whether they are all substantially and in essence similar or dissimilar. Tinctorially the three show independently variable chromophilia and behave differently under varying circumstances. The bridges become prominent in relatively slow hyperplasia, such as after M. C. A., and reach their greatest prominence in carcinoma produced by this drug. On the other hand, the peripheral fibril, normally the principal tonofibrillar component of basal cells, shows especial prominence in the acute and florid phases of podophyllin activity. The marked peripheral prominence gradually regresses with the reparative phase.

These three components of the tonofibrillar mass may represent different functional responses of the cell, correlated with differing metabolic activities. The details of these activities are as yet unknown. Certain facts stand out clearly. Tonofibrils, very scanty in normal mouse skin, undergo rapid hypertrophy and proliferation under conditions of stimulation. If the stimulus is towards simple hyperplasia of epidermis (as from M. C. A. applications), an end result is produced reminiscent of the fibrillar pattern of normal human skin. If, however, the stimulus is the action of podo-

phyllin, the reaction is more complex. The peripheral fibril (or spiral of Herxheimer) is the first to enlarge and become prominent, and is observed not only in the basal layer, but throughout the entire epidermis. More centrally placed fibrils become prominent only as a later development, while intercellular bridges also show only a late appearance. The tonofibrillar development takes place despite severe swelling and pallor of the cells and in spite of prolonged arrested mitosis or the transformation of nuclear fragments into micronuclei. In other words, neither integrity of the nucleus nor the normal basophilia of the cytoplasm is required for the production of tonofibrils. The fibrils are abundantly produced in spite of severe morphologic disturbances of cytoplasm.

Yet in cells swollen and altered under the effect of podophyllin, the fibrils are wavy, tortuous, and rather lacking in the usual distinct orientation seen in other circumstances. The relationship of tonofibrils to keratinization is difficult to decipher. The scanty recent literature, as reviewed above, indicates a definite connection, and our own observations, especially on carcinomas, support this concept. Yet other observations appear to be at variance. For example, the problem has already been touched upon, that where keratohyaline granules are abundant, in cells immediately prior to keratinization, the fibrils are scanty.

A suggestion may be brought forward, that there are many different kinds of keratin. We need not be concerned here with the forms A, B, and C of Unna, nor α and β of Astbury. Mention may be made of a previous study (*20*) on epidermal cysts and their occasional mummification. Here it was pointed out that different varieties of keratin must be postulated, on the basis of their appearance and behavior, a difference which may be very marked. Similarly, in one of the earlier studies on podophyllin, differences in behavior and origin of certain keratinizations were suggested. Reasonable doubt may be expressed whether X-ray studies are sufficiently delicate to resolve the problem. Histochemical analysis, as well as pure morphological observations, is needed on such diverse forms of keratinization as are seen in condylomata, verrucae, the wide variety of epidermal cysts, including the mummified form, dermoid cysts, parakeratosis, experimental and natural carcinogenesis, and the like. The literature has merely touched on the problem principally from the standpoint of anatomy, while the wide field of pathology has barely been scratched. Such an approach is outside the immediate scope of these studies on podophyllin.

The fact that some forms of keratin stain with P. T. A. in an intense, essentially homogeneous manner, while in other forms the keratin exhibits an intensely fibrillar character, is not easily explained. Technical methods, as emphasized in the literature, may play some part. But with a standardized technique, as used herein, the difference cannot be ignored. Speculation may be offered, without proof, that keratin molecules undergo a process of polymerization and depolymerization; that one or more stages of the process is associated with tonofibrils but that there is no

1:1 correlation; that the rapidity or degree of the polymerization (or depolymerization) is a factor controlling variations of appearance in differing types of keratin.

CONCLUSION

There are three distinguishable types of tonofibrils in squamous epithelium of mouse skin. These three types are probably basically similar but reveal variable prominence according to the functional state of the cell.

Tonofibrils are very few in normal mouse epidermis. Under the influence of podophyllin, fibrils are produced in great numbers, with appearance and distribution characteristic for early and late stages. The formation of tonofibrils occurs in spite of severe morphologic disturbances of the cytoplasm. Integrity of the nucleus is not required for such formation. Intense production of tonofibrils takes place in cells with arrested mitoses and with micronuclei.

In hyperplasia (or acanthosis) of epithelium caused by methylcholanthrene, tonofibrils multiply in a regular orderly fashion. Subsequent application of podophyllin alters the course and appearance of the fibrils, producing a picture comparable to that seen following podophyllin applied to normal skin.

Tonofibrils are a stable constituent of epidermal cytoplasm, although labile in respect to chromophilia and arrangement.

In carcinogenesis, tonofibrils are very prominent in well-differentiated tumors but show a pattern different from that seen in simple acanthosis following podophyllin.

Tonofibrils appear to be intimately associated with keratin and the process of keratinization. Evidence suggests that there are many different varieties of keratin, above and beyond the analyses of Unna and of Astbury. It is possible that tonofibrils represent a stage in the polymerization of keratin molecules.

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PLATE 99

FIGURE 1.—Phosphotungstic acid stains, oil immersion photographs, magnification of approximately ×1,100. In all pictures of this plate the free surface of the skin is on the left, the corium on the right.

A. Early effect of podophyllin acting on normal skin. The cells are ballooned and swollen and show at the periphery a sharply defined deep-staining fibril, suggestive of a cell membrane but actually constituting the peripheral Herxheimer fibril. The arrows pointing to the left indicate cells where the fibril is especially sharply focused. The arrow pointing to the right indicates a "podophyllin cell," with distorted mitosis, revealing a sharp peripheral cytoplasmic fibril, but no nuclear membrane.

B. Late podophyllin effect. A large "podophyllin cell" is seen with broken up chromatin material, and concentric wavy peripheral fibrils, and many delicate cross-fibrils. Cells in the upper portion show considerable tonofibrillar development, but the peripheral Herxheimer fibrils, prominent in the early phases, are no longer conspicuous.

C and *D.* Podophyllin effect, florid stage. The cells are larger than in the early stage, and still vacuolated. The peripheral fibrils are still prominent, but within the spongy cytoplasm can be seen an irregular meshwork of delicate intracellular fibrils. Intercellular bridges are inconspicuous, although intercellular spaces are frequently distinct. In *C*, the arrows point to Herxheimer fibrils which penetrate into the corium.

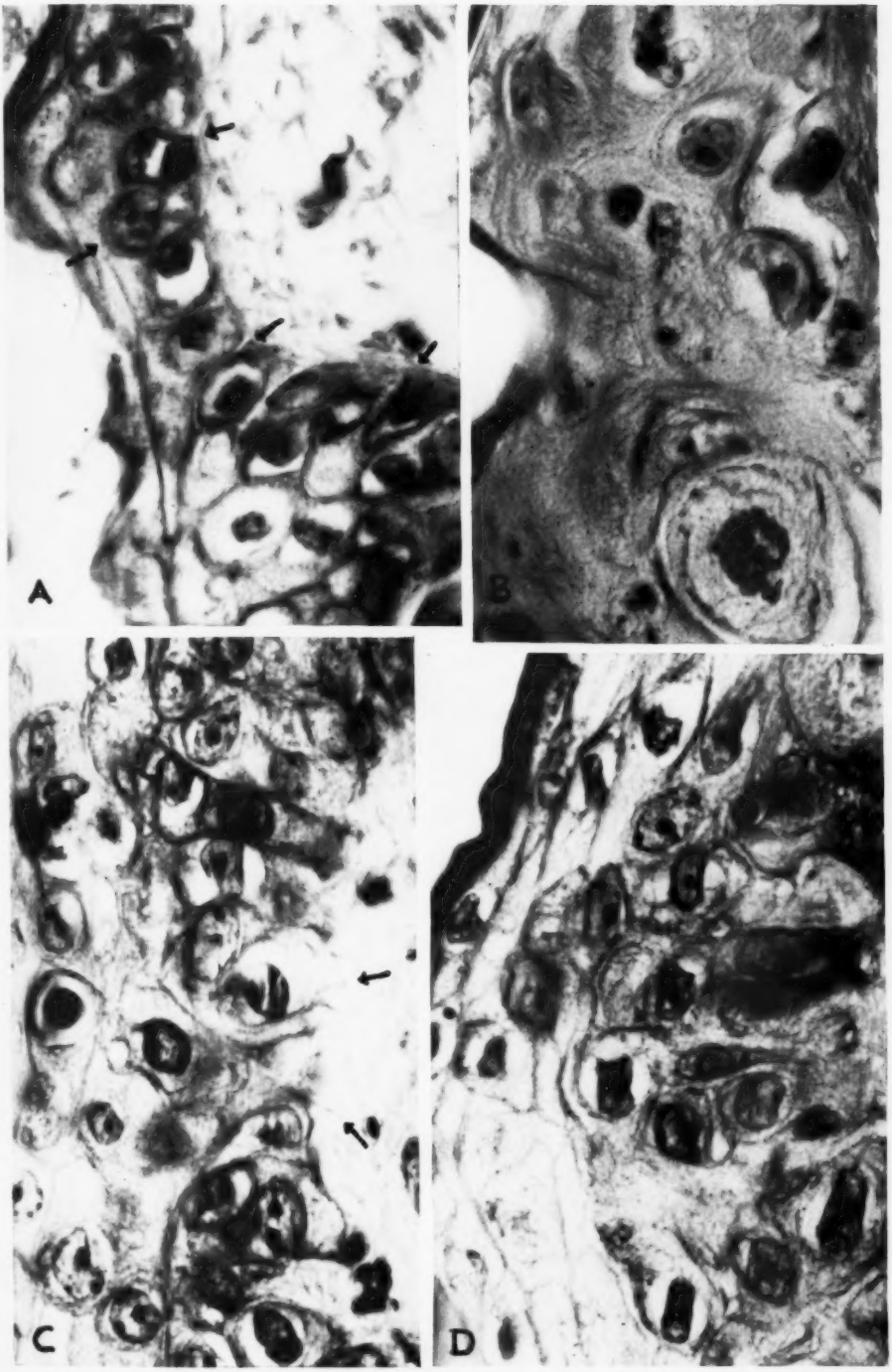
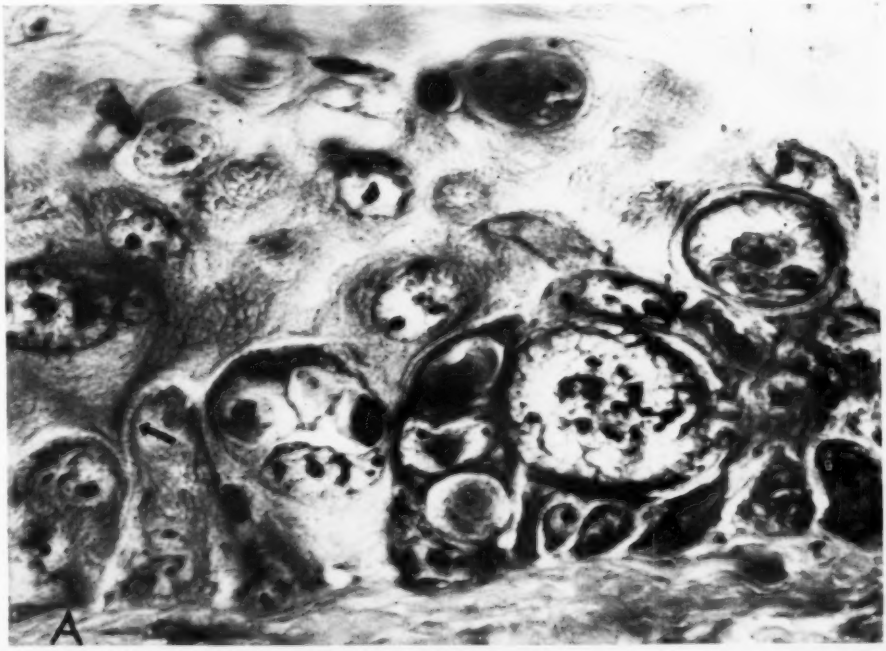

A
B
C
D

PLATE 100

FIGURE 2.—Phosphotungstic acid hematoxylin, oil immersion photographs, magnification approximately ×1,100.

A. Late podophyllin effect. A large tetraploid "podophyllin cell" shows dispersed chromatin fragments and a heavy meshwork of coarse deep-staining fibrillar strands within the cytoplasm. There is also a large cell with four micronuclei, with heavy tonofibrillar strands radiating inward from a prominent peripheral fibril. By contrast the cells on the left reveal well developed intracellular fibrils with minimal peripheral accentuation. The arrow points to some intercellular bridges. This figure, contrasted with figure 1, *A*, shows well the difference between early and late podophyllin effect.

B. Acanthosis, or hyperplasia, produced by three applications of methylcholanthrene. The epidermis is broadened and shows differentiation into basal, prickle, and granular layers. The tonofibrils are very well developed, with slight peripheral accentuation. The fibrils pursue a regular well-defined course. Intercellular bridges are distinct. This type of nonspecific acanthosis is also seen in the late reparative stage following podophyllin application, when specific podophyllin effect is no longer apparent.

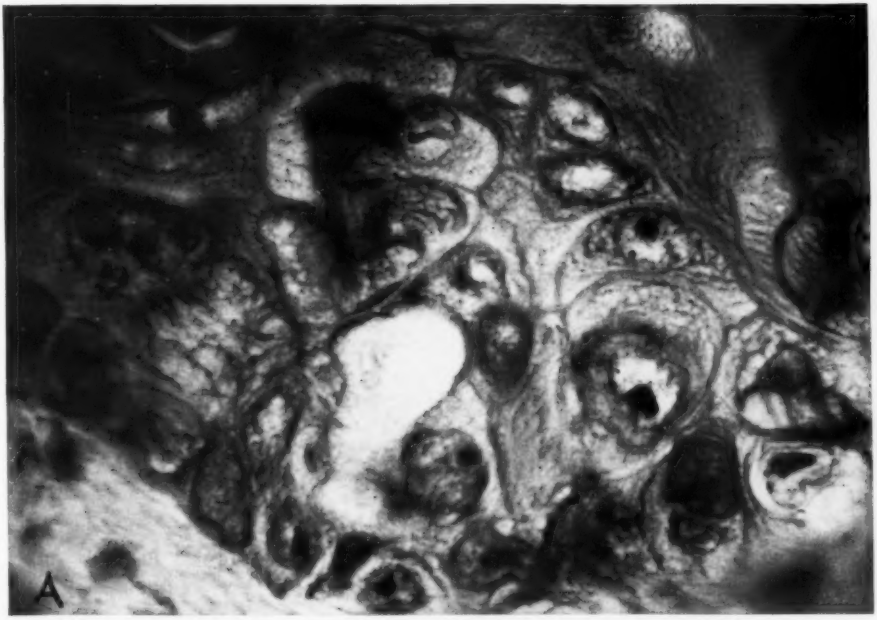

A

B

PLATE 101

FIGURE 3.—Phosphotungstic acid stain, oil immersion photographs, magnification approximately × 1,100.

A and *B*. Effect of podophyllin applied not to normal mouse skin but to skin made acanthotic by methylcholanthrene (such as is seen in fig. 2, *B*). *A* is 48 hours and *B* is 24 hours after podophyllin treatment. The tonofibrillar pattern seen in figure 2, *B* has been distorted and modified and resembles the results produced by podophyllin on normal skin. However, there has been acceleration effect, so that the 24-hour action on acanthotic skin resembles the 48-hour effect on normal skin.


A

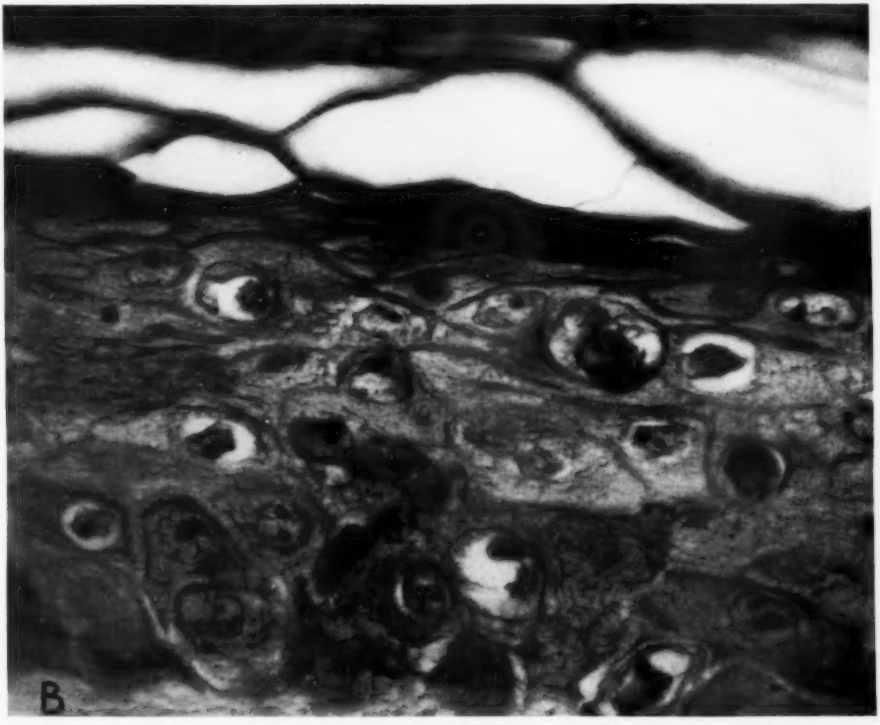

B

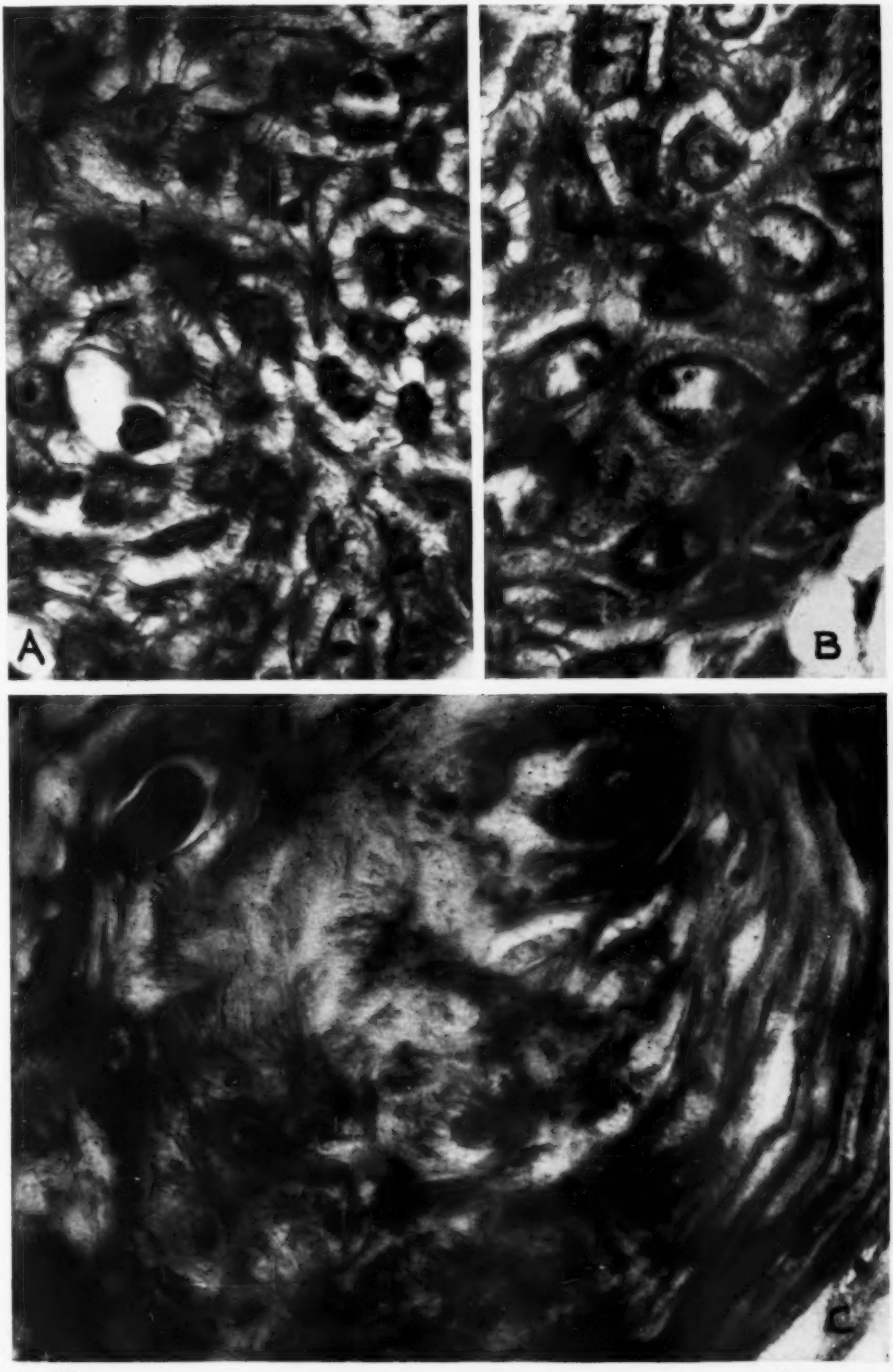
PLATE 102

FIGURE 4.—Phosphotungstic acid stain, magnification approximately × 1,100. Tonofibrillar picture of squamous carcinoma produced by methylcholanthrene.

A. reveals the very prominent and intense development of the intercellular bridges. The arrow indicates nodal points, especially well shown here. The intracellular fibrils, however, are relatively scanty.

B. Another field from the same slide showing a somewhat greater development of intracellular fibrils. The arrow points to a cell densely crammed with fibrils. The perpendicular orientation of the intercellular bridges is clearly seen.

C. An epithelial pearl in a squamous carcinoma. The keratinizing material reveals a heavy feltwork of distinct fibrils, whereas mature surface keratin generally appears homogeneous and nonfibrillar (cf. figs. 1, *D*, 2, *B*, or 3, *B*).


A
B
C

ECLAMPSIA-LIKE CONDITION IN PREGNANT RATS INJECTED WITH PROGESTERONE [1] [2] [3]

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Previous experiments have demonstrated that progesterone in large dosage injected into mice in late pregnancy has a lethal action upon both mothers and embryos. This treatment was followed by abortion or resorption of the embryos and by death of approximately 40 percent of the mothers during or shortly after treatment (*1*, *2*, *3*).

The present experiments were carried out, using rats, in order to determine if possible the pathogenesis of this action of progesterone in late pregnancy and to observe the attendant and later effects of the hormone injection on the organs of the surviving rats.

MATERIALS AND METHODS

The experiments were carried out with four series of rats of the inbred Marshall-520 strain. The animals were about 3 to 4 months of age when the observations were started.

Series I. Twenty-four females were mated with males of the same strain. The animals were checked twice a day for vaginal plugs. On the 15th, 16th, 17th, and 18th days of pregnancy, each of 20 animals was injected intramuscularly with 5 mg. of progesterone daily for a total dosage of 20 mg. of progesterone.[4] Each of four rats was injected with 10 mg. of progesterone on the 16th and 17th day of pregnancy again totaling 20 mg. of progesterone per animal.

Series II. Each of 10 female rats was injected with 5 mg. of progesterone daily for 4 days from the 10th through the 13th day of pregnancy.

Series III. Each of 10 female rats was injected with corn oil daily from the 15th through the 18th day of pregnancy.

Series IV. Each of 10 virgin female rats was treated daily for 4 days with 5 mg. of progesterone.

All animals were fed Purina laboratory chow pellets and given an unlimited supply of drinking water during the course of the experiment.

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[2] Presented before the American Society for Experimental Pathology, April 1949, Detroit, Mich.
[3] This investigation has been aided by a grant from the Jane Coffin Childs Memorial Fund for Medical Research.
[4] Crystalline progesterone dissolved in corn oil was used in this experiment.

RESULTS

In Series II and III, none of the treated animals died. They appeared to be healthy, and when autopsied no lesions were found. The pregnant females which were treated before the 15th day of pregnancy delivered normally. The biologic activity of the injected progesterone at the dose employed was established by producing large deciduomas in four virgin females in series IV, which otherwise showed no significant change and appeared healthy clinically.

In Series I, in which the animals were treated in the last third of pregnancy, a period of obvious illness followed the injections. The animals lost their normal vigor and activity; the coats were roughened, and the eyelids were swollen; movements were slow. Cyanosis of the extremities and vaginal bleeding were noticed in all the very sick animals. Eight rats recovered following abortion of dead embryos between the 22nd and 24th day following the onset of pregnancy. In six rats the embryos were resorbed. Three rats died and seven rats were killed when moribund, between the 20th and 23rd day following the onset of pregnancy. The surviving animals are being kept for further observation.

Examination of the urine in 15 rats revealed albuminuria (1+ to 3+). In 7 animals which were killed when moribund, heart blood was taken for determination of the nonprotein nitrogen. In all these rats a retention of nonprotein nitrogen (NPN) (69.8, 74, 80.4, 87, 106, 110, 120, and 160 mg./100 cc. in blood serum) was found. The systolic blood pressure was significantly elevated in all the 5 animals examined with the plethysmograph (190–220 mm. Hg).

PATHOLOGICAL FINDINGS

The pathological findings are based on the 10 autopsied animals from Series I. The lesions were constant throughout this series and can be summarized as follows:

Grossly, the most striking and characteristic changes were found in the liver, the kidney, and the placenta. The dead embryos in the uterus were well developed and in varying stages of maceration. The placentas were pale yellow-grey and were attached to the uterine wall. They were usually surrounded or covered with large, fresh blood clots (Fig. 1). The lungs were edematous and partially atelectatic, compressed by a clear transudate in the pleural cavity. The adrenal glands were hemorrhagic in two cases. The thymus gland and the thyroid gland were reduced in size. The liver was enlarged and showed a varying picture. In some cases it was mottled reddish-brown with scattered red and yellow areas. In other cases it was greyish-yellow with red areas. The kidneys were slightly enlarged, with a smooth surface and mottled pink, grey, and yellow (Fig. 2).

Microscopically, the liver showed areas of necrotic hepatic cells located predominantly in the periphery of the lobules. The cell nuclei were

pyknotic, and there was mummification of the necrotic liver-cell cords (Fig. 3). Fatty change of the hepatic cells was found to vary in intensity in different cases. In all the animals examined the disappearance of glycogen from the liver cells was a constant finding. The liver-cell degeneration appeared to start with intracellular hyaline droplet accumulation (Fig. 3). Dilatation of the capillary sinusoids, fibrin thrombi, and a few hemorrhagic foci gave a striking resemblance to the human eclamptic liver (Figs. 4 and 5).

The kidneys showed striking microscopic lesions. In severely affected animals, diffuse glomerular changes were found. In general, the involved glomeruli were enlarged, and fibrin thrombi were observed in segments of the glomerular loops (Fig. 6), often propagating into the afferent arteriole and less often as far as the intralobular artery (Fig. 7). Exceptionally, the thrombus extended as far as the efferent arteriole (Fig. 8). Most of the involved glomeruli were completely ischemic, and others were partially so.

Changes were observed in the basement membrane of the capillaries of the glomeruli. This alteration consisted of irregularity in outline, increased thickness, and fibrillation (Fig. 10). This was well demonstrated with the periodic-acid stain (Figs. 9 and 10). In frozen sections stained for fat, pronounced fatty degeneration of the endothelium of the glomerular tuft and the arterioles was demonstrated (Fig. 11). These glomerular lesions appeared to be degenerative in nature. The tubular epithelium likewise showed degenerative changes with extensive areas of necrosis and other areas of hyaline droplet degeneration (Fig. 12). Slight fatty degeneration could be demonstrated by staining with scarlet red. Many casts were found in the collecting tubules.

In comparison with the normal placenta (Fig. 13), the lesions of the placenta in the treated animals consisted of a degeneration of the cellular chorionic elements (Fig. 14). The Langhans cells disappeared, and the syncytium showed varying stages of degeneration, atrophy, and necrosis (Fig. 15). The blood vessels were dilated, and there was extensive periplacental hemorrhage.

The adrenal glands showed no remarkable lesions with the exception of two cases with hemorrhagic necrosis of the cortex.

The thymus gland showed conspicuous changes consisting of nuclear pyknosis and almost complete dissolution of the thymocytes. The lymphatic tissue of the spleen and of the lymph nodes was also affected similarly but to a less pronounced degree than the thymus.

The brain was edematous. Possible changes in the pituitary gland will be studied more fully and described later.

DISCUSSION

Many attempts have been made to produce the lesions and the syndrome of eclampsia experimentally in animals. Experiments with extracts of the posterior lobe of the hypophysis have been carried out using

pregnant and nonpregnant females and male guinea pigs and rats, Fauvet (*4*), Vegh and Pallos (*5*), Byrom (*6*). With very high doses of vasopressin, degenerative lesions in the walls of the large blood vessels with areas of necrosis of the parenchyma in the kidney and liver have been induced. Elevation of blood pressure was noticed as an effect of the vasopressin. Oligmacher (*7*) examined the experimental material of Fauvet and repeated his experiment. He found that the induced kidney and liver lesions in his experimental animals as well as in those of Fauvet had no similarity to those of human eclampsia. Oligmacher states that Fahr examined the same slides and confirmed his findings.

Constriction of the renal arteries in pregnant dogs and rabbits induces renal necrosis and liver lesions which have been described as resembling those of human eclampsia (*8*).

Smith and Smith (*9*) have injected nonpregnant rats with menstrual discharge and induced "parenchymatous degeneration, congestion and often capillary hemorrhage" in the kidney as well as degenerative changes in the liver. These authors consistently found hemorrhage and dissolution of cells in the adrenal glands. It is interesting that Smith and Smith (*10*) found that the menstrual discharge has progesterone-stimulating effect in rats.

Greene (*11*) has produced eclampsia-like lesions in pregnant rabbits both by injecting an aqueous extract of the anterior lobe of beef pituitary and by antuitrin. The induced clinical symptoms and lesions were similar to those which Greene (*12*) had previously described in an eclampsia-like disease occurring spontaneously in rabbits in which "blood spots" in the ovaries were reported. The question raised in interpreting these results is whether the hypophyseal extract has not overstimulated the corpora lutea with a hypersecretion of progesterone.[5]

Dexter and Weiss (*13*) injected progesterone in four nonpregnant rats and in two pregnant rats in late pregnancy. Their doses were small and results negative. Selye and collaborators (*14*) injected into nonpregnant rats 5 mg. of progesterone daily for 20 days. They found no lesions in the kidney.

That high doses of progesterone in late pregnancy have a lethal action upon embryos has been confirmed by the experiments of Ehrhardt and Koenig (*15*). However, these authors do not give enough information about the effect on the mothers.

The present experiment demonstrates that an eclampsia-like condition can be produced with an ovarian hormone, progesterone. This effect is produced only when the animals are pregnant, and the hormone injections

[5] During the discussion of the present paper at the meeting of the American Society for Experimental Pathology, Dr. Max B. Lurie mentioned a similar lethal eclampsi-alike disease occurring in late pregnancy in an inbred strain of rabbits in his laboratory. He pointed out that at autopsy the ovaries of these animals were found consistently enlarged with remarkably large corpora lutea. A similar disease with the pathological and ovarian findings in an inbred strain of guinea pigs was also mentioned.

are effective in late pregnancy. In respect to time, therefore, this condition corresponds to the human disease, and other clinical symptoms of eclampsia, namely, albuminuria, azotemia, edema, and hypertension, correspond to those accompanying toxemia of pregnancy in the human female. The high NPN retention corresponds rather to the cases of eclampsia with severe kidney lesions. The lesions in the liver and the kidney are similar to those of human eclampsia grossly as well as microscopically in its typical form as described by Fahr (*16*). The liver shows peripheral lobular necrosis with capillary dilatation, fibrin thrombi, and a few hemorrhages. Fibrin thrombi in glomeruli, thickening of the glomerular basement membrane, tubular degeneration, and necrosis are the lesions of the kidney.

The degenerative and necrotic lesions of the trophoblastic cells found in the placenta are of great interest because of the importance attributed to similar lesions in human eclampsia (*17*). Because of species differences in placentation, one should not expect placental lesions which correspond exactly to those seen in human eclampsia. This point needs further comparative study.

It is also of interest to compare the lesions induced in the present experiment with those described by Selye (*18*) as an alarm-reaction; however, the lesions of our animals have specific characteristics such as the fibrin thrombi in the sinusoids of the liver, the glomeruli of the kidney, and the thickening of the glomerular basement membrane.

In animals with severe kidney damage there developed a condition similar to cortical necrosis as seen in the human female. The degenerative and necrotic lesions of the tubules were scattered throughout the cortex surrounding the damaged glomeruli. Some authors, Bell (*19*), and Dunn and Montgomery (*20*), have stated that although renal cortical necrosis may be classified with the toxemias of pregnancy, it is probably not the same disease as eclampsia. The prevailing opinion is that renal cortical necrosis in pregnancy should be considered not as an independent disease but as a complication of eclamptic toxemia (Schmorl (*21*), Fahr (*16*), Dexter and Weiss (*13*)).

At this stage of these experiments, it is not possible to make any correlation between the etiology and pathogenesis of this experimentally induced syndrome and human eclampsia, but it is of more than passing interest that clinical investigations have demonstrated that an abnormality in the progesterone excretion may be present in human eclampsia. Hain (*22*) found an increase of progesterone in eclampsia. Smith and Smith (*23*) claim, on the basis of their outstanding clinical work, that toxemia of late pregnancy is due to the withdrawal of hormone support from the pregnant uterus which results in the formation of a menstrual-like toxin. The release of this toxin may be the final precipitating cause of the eclampsia.

At present three possible hypotheses are considered concerning the action of progesterone in the production of this particular experimentally induced eclampsia-like syndrome in rats:

(1) High doses of progesterone produce hormonal imbalance at a critical stage of late pregnancy which leads to a chain of reactions resulting in an eclampsia-like syndrome.

(2) Progesterone damages the embryos and placentas resulting in the production of toxic substances. The fact that some animals have recovered after discharging dead embryos and damaged placentas is compatible with this hypothesis.

(3) High doses of progesterone have a direct toxic action, *per se*. If this is the case, then it is interesting that the toxic action is manifested only in pregnant animals, and only in the last third of pregnancy.

The important points in the above mentioned hypotheses and the correlation among them are at present under investigation.

SUMMARY

An eclampsia-like syndrome has been induced in rats by the injection of large doses of progesterone during their last third of pregnancy.

Death, abortion, or resorption of the embryos as well as obvious illness and death of the mothers followed the treatment.

Albuminuria, azotemia, edema, and hypertension were found in the treated rats.

The lesions in other organs, especially in the liver and the kidney, were similar to those of human eclampsia. These were peripheral lobular necrosis of the liver, with capillary dilatation, fibrin thrombi, and hemorrhages, fibrin thrombi in glomeruli, thickening of the glomerular basement membrane, and tubular degeneration and necrosis in the kidney.

Control series of pregnant rats prior to the last third of pregnancy and nonpregnant rats receiving the same treatment failed to show any similar changes.

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PLATE 103

FIGURE 1.—Pregnant rat sacrificed when moribund with dead embryos, periplacental hemorrhages (bluish areas), and congested and swollen liver.

FIGURE 2.—Enlarged, reddish-brown liver with multiple red areas scattered throughout. Slightly enlarged kidneys with mottled pink, grey, and yellow cortex and hyperemic medulla.

FIGURE 3.—Hyaline droplet degeneration with beginning necrosis and mummification of liver cell cords at the periphery of lobules (periodic-acid stain, photomicrograph × 125).

FIGURE 4.—Liver-cell necrosis, dilatation of the capillary sinusoids, with red stained fibrin thrombi. Absence of glycogen in the liver cells (periodic-acid stain, photomicrograph × 125).

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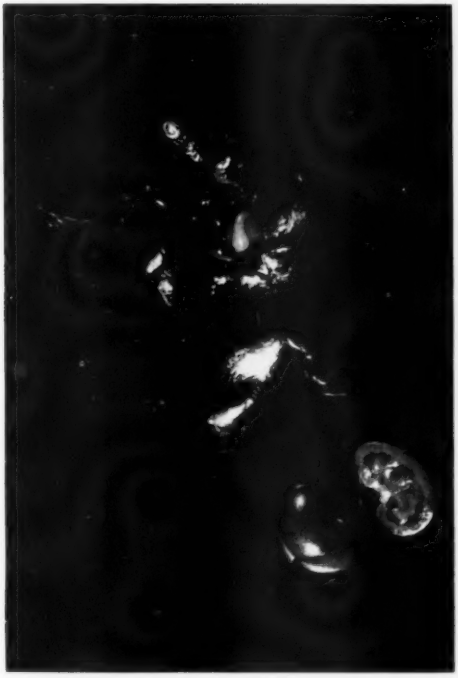

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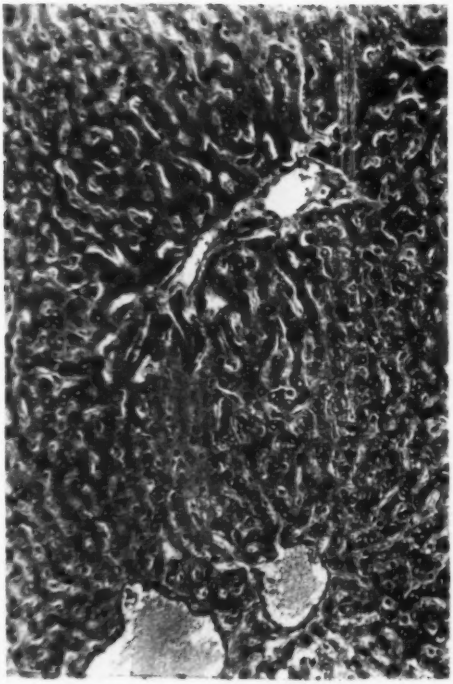

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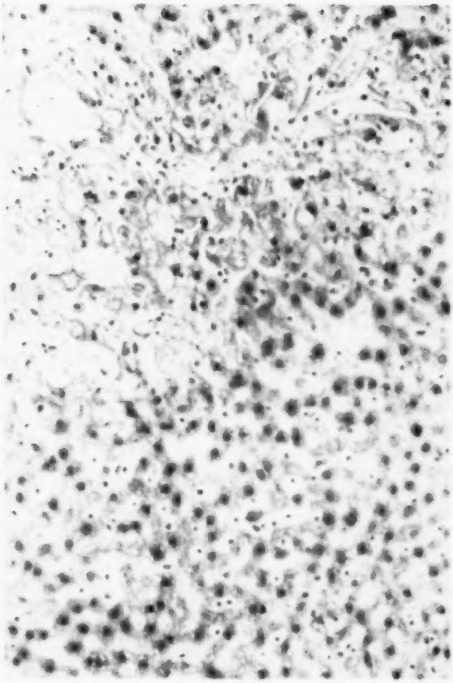
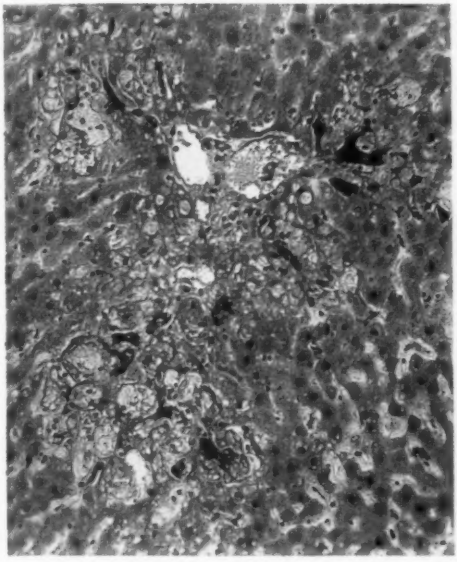


PLATE 104

FIGURE 5.—Dilated capillary sinusoids at the periphery of lobules of the liver. Fibrin thrombi (violet stained) in the capillary with hemorrhage and liver-cell degeneration (Gram-Fibrin and Carmin counterstain, photomicrograph, × 200).

FIGURE 6.—Fibrin thrombi (red stained) in the capillaries and the afferent arteriole of glomeruli. Degeneration and necrosis of the tubules (periodic acid stain, photomicrograph, × 200).

FIGURE 7.—Red stained fibrin thrombus extending from a glomerular loop into the dilated afferent arteriole to the intralobular artery. Degeneration and necrosis of the kidney tubules surrounding the glomerulus (periodic acid stain, photomicrograph, × 395).



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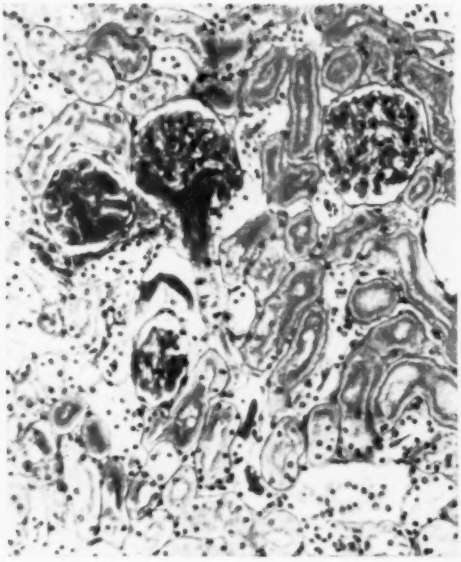

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PLATE 105

FIGURE 8.—Fibrin thrombi (stained blue) in the glomerular capillaries extending to afferent and efferent arterioles (Gram-Fibrin and Carmin counter stain, photomicrograph, × 395).

FIGURE 9.—Glomerulus of a normal kidney of an untreated rat in late pregnancy. Thin, clearly outlined basement membrane of capillaries containing blood (periodic acid stain, photomicrograph, × 435).

FIGURE 10.—Ischemic glomerulus, showing thickening, fibrillation and irregularity in outline of the basement membrane (periodic acid stain, photomicrograph, × 475).

FIGURE 11.—Fatty degeneration of the endothelium of the glomerular capillaries and the afferent arteriole (frozen section, scarlet red stain, photomicrograph, × 475).

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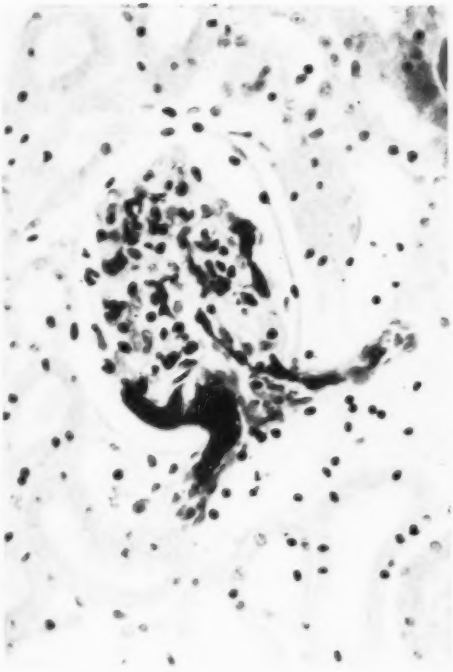

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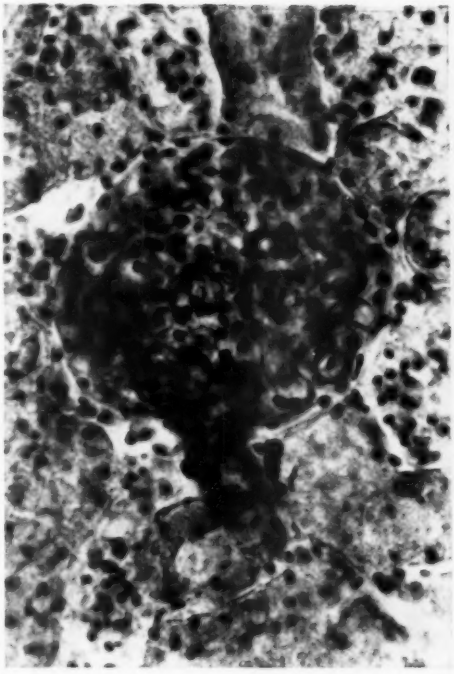

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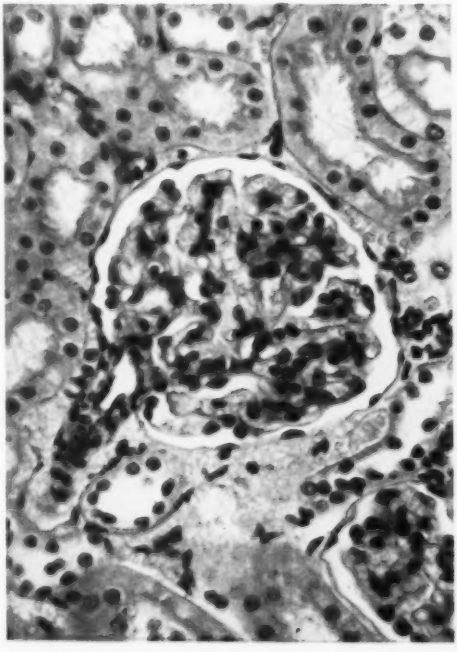

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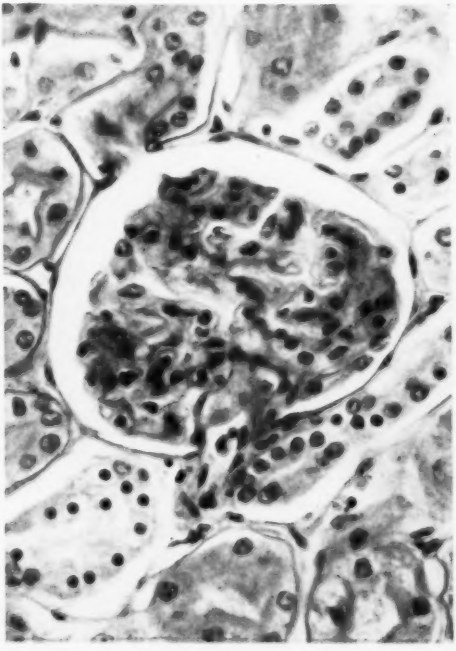

PLATE 106

FIGURE 12.—Degenerative lesions of the tubular epithelium of the kidney. Slight degenerative changes in the left part, with hyaline droplet degeneration of epithelial cells in the middle portion and necrotic lesions in tubules of the right part (periodic acid stain, photomicrograph, × 125).

FIGURE 13.—Placenta of a normal nontreated rat. Langhans cells in the center contain red staining glycogen with dark peripheral syncytial cells (periodic acid stain, photomicrograph, × 125)

FIGURE 14.—Pale trophoblastic cells in an early degenerative stage (periodic acid stain, photomicrograph, × 125).

FIGURE 15.—Abnormal degenerating trophoblastic cells at the periphery of necrotic placental tissue (periodic acid stain, photomicrograph, × 125).

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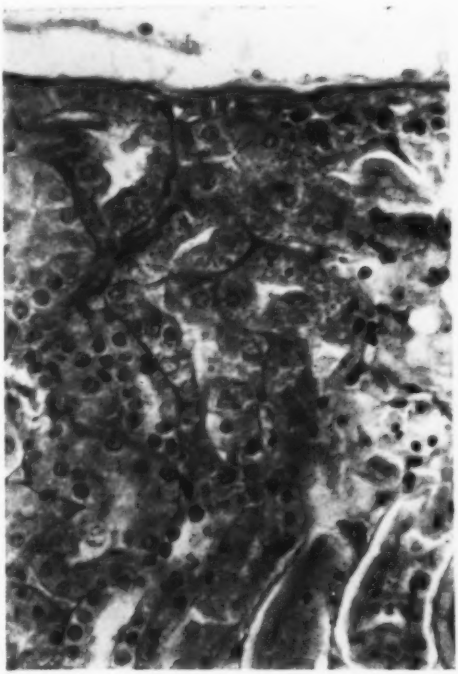

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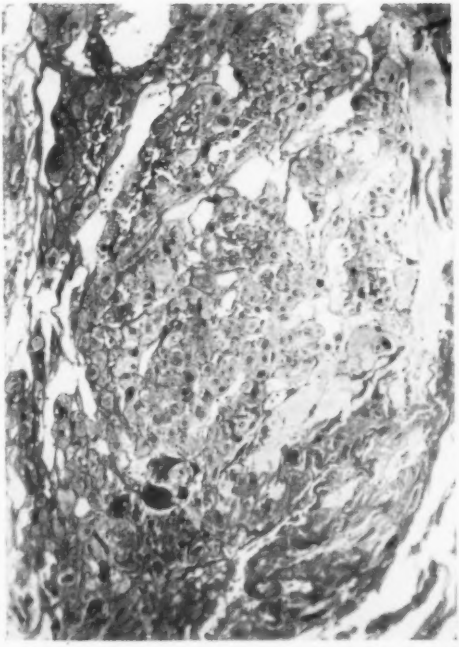

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THE ANTIPROTEOLYTIC REACTION, IODOACETATE INDEX AND LEAST COAGULABLE PROTEIN REACTIONS IN RELATION TO MALIGNANT NEOPLASIA: CORRELATION OF TESTS [1] [2]

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The search for a specific general test for the presence of malignant neoplasia has been diligent but without avail. All such tests so far reported have been nonspecific in nature and suffer from all the disadvantages of numerous false negative and false positive reactions. Manipulation of the test, or the combination of tests, to make them more sensitive for diagnosis of malignant tumor, have been successful in that respect (*1*, *2*, *3*) but they then become more sensitive to the diseases which give false positive reactions.

Huggins, *et al.* (*2*), with their modification of the Protein Coagulation Test for malignancy, observed a very high correlation between the I. A. I. (iodoacetate index) and the presence of malignant neoplasia and a very few false positive reactions. They also reported no false positives in pregnancy (*3*), which in other tests had given a very high percentage of false positive reactions (*4*). Since these results were so far superior to our results with the A. P. L. (antiproteolytic reaction) (*4*), we felt that a correlation of the three reactions with our own patient material would be desirable in determining whether we should abandon work on the A. P. L. reaction.

METHODS

Simultaneous tests were made, using aliquots from the same serum sample. Repeat examinations of the I. A. I. and L. C. P. (Least Coagulable Protein) were made in approximately 20 percent of the cases, and good checks were obtained, so far as positive and negative results were concerned.

The method for determination of the antiproteolytic titre was identical with that reported previously (*4*), and the reported method (*2*) for determining I. A. I. and L. C. P. was used in the early tests and subsequently

[1] Received for publication October 13, 1949.
[2] This study was supported by the Rockford Fund of the Yale University School of Medicine.
[3] This work was carried out in part during tenure as a Senior Fellow of the American Cancer Society.

the modified method, as suggested from Huggins' laboratory, was used although we found no significant differences in the results. All tests were run as routine examinations by specially trained technicians. Since the I. A. I. and L. C. P. results are reported positive and negative only, we revised the method of reporting the A. P. L. tests (*4*) so that all were reported as positive or negative with 130 percent of normal being the dividing line (130 percent and above, positive; 129 percent and below, negative).

All diagnoses of carcinoma were made by tissue section, although this was not repeated in all recurrent cases. All patients have been carefully and repeatedly checked to establish the diagnosis, and all have been followed for a minimum of 2 months.

Seven patients of the 260 studied remain unsatisfactorily diagnosed because of refusal of treatment or biopsy or because of inadequate follow-up. These are excluded from the results. Of the remaining 253, not all patients had all tests performed. Two hundred and fifty-three patients had A. P. L. tests, 251 had L. C. P. tests, and 225 had the I. A. J. of their serum determined. All had either L. C. P. or I. A. I.

The patients are divided into 11 groups: Group 1, those with malignant neoplasia whether primary or recurrent and with or without metastases; Group 2, all patients who have had definitive treatment for carcinoma in the past and now show no evidence of recurrence; Group 3, all pregnant patients, and incidentally, all were at least in the fourth month of their pregnancy; Group 4, those with tuberculosis minimal to advanced, and in all parts of the body; Group 5, those with acute infections; Group 6, those with chronic infections including several patients with healing sinuses; Group 7, those with peptic ulcer whether duodenal or gastric, with or without evidence of activity, with and without hemorrhage; Group 8, postoperative patients (of which only three were tested); Group 9, those with all other diseases including diabetes, goitre, cardiac disease, hepatic disease, etc., in all stages of severity; Group 10, all patients with benign tumors; Group 11, all patients without apparent disease (N. A. D.). The patients with and without malignant neoplasia fall in the same age groups.

RESULTS

In table 1, the cases are divided into the 11 categories previously mentioned, and the total number of positive and negative results plus the percentage of correct results in each group are recorded. It is obvious that the great majority of false positive reactions fall into the pregnancy, tuberculosis, acute infections, and postoperative groups. In table 2 the results are recorded as correct or incorrect in relation to the diagnosis in each group, and the ratio of correct to incorrect results of the total group is determined.

From these tables it is clear that the I. A. I. is positive in most cases of malignancy (83.3 percent), the L. C. P. is positive in 78.3 percent, and the A. P. L. is positive least frequently (70 percent). However, when one

considers other diseases, it is seen that the A. P. L. titre is negative when it should be in a much higher percentage of cases than are either of the other tests except in one category, pregnancy. For overall accuracy of the test in this series of cases the A. P. L. test is found to be correct in 75 percent of cases, the I. A. I. is correct in 55.5 percent, and the L. C. P. is correct in 67.6 percent of cases studied.

TABLE 1

Group	A. P. L.			I. A. I.			L. C. P.		
	+	−	Correct	+	−	Correct	+	−	Correct
			Percent			*Percent*			*Percent*
1. Malignant tumor	42	18	70	45	9	83. 3	47	13	78. 3
2. No evidence recurrence malignant tumor	3	17	85	9	11	55	9	11	55
3. Pregnancy	17	0	0	14	3	17. 7	10	7	41. 2
4. Tuberculosis	6	6	50	11	1	8. 4	7	5	41. 7
5. Acute infections	6	0	0	4	0	0	5	1	16. 7
6. Chronic infections	2	17	89. 5	11	8	42. 1	7	12	63. 2
7. Peptic ulcer	5	10	66. 6	12	3	20	6	9	60
8. Postoperative	2	1	33. 3	3	0	0	2	1	33. 3
9. All other diseases	1	36	96. 3	19	16	45. 7	13	22	62. 9
10. Benign tumor	1	16	94. 2	8	6	42. 9	5	12	70. 6
11. No apparent disease	2	45	95. 75	10	32	77. 2	4	43	91. 5

TABLE 2

Group	A. P. L.		I. A. I.		L. C. P.	
	Correct	Incorrect	Correct	Incorrect	Correct	Incorrect
1. Malignant tumor	42	18	45	9	47	13
2. No evidence recurrence malignant tumor	17	3	11	9	11	9
3. Pregnancy	0	17	3	14	7	10
4. Tuberculosis	6	6	1	11	5	7
5. Acute infections	0	6	0	4	1	5
6. Chronic infections	17	2	8	11	12	7
7. Peptic ulcer	10	5	3	12	9	6
8. Postoperative	1	2	0	3	1	2
9. All other diseases	36	1	16	19	22	13
10. Benign tumor	16	1	6	8	12	5
11. No apparent disease	45	2	32	10	43	4
Total	190	63	125	110	170	81
Percent correct	75	------	55. 5	------	67. 7	------

DISCUSSION

Since all tests for malignancy so far suggested are nonspecific, great care must be taken in clarifying the false positive and false negative reactions, and the range of positive and negative must be so established as to give the most satisfactory results. Although it is desirable to obtain as many positive tests as possible in the presence of malignancy, it must

also be recognized that overloading with false positive reactions, especially if they be in patients without other serious diseases will result in needless and, indeed, hopeless attempts to establish a diagnosis of malignancy in large numbers of people without disease. From the point of view of detection of malignancy, this would tend to further overload the already taxed machinery of detection rather than to decrease the load.

By lowering the range of positive for the A. P. L. reaction from 130 percent to 125 percent of normal, it would be possible to increase the number of correct positives in malignant neoplasia from 42 of 60, or 70 percent, to 50 of 60, or 83.3 percent, the same result as with the I. A. I. With this increase in correct diagnosis there is, however, a significant increase in the number of false positive reactions in other diseases, and the overall accuracy of the reaction drops to 67.1 percent, essentially the same as the L. C. P. results.

With combinations of tests giving false positive reactions it seems likely that a similar shift will occur with an increase in correct diagnosis of malignancy and an overall decrease in accuracy of diagnosis. Combining these 3 tests and using a positive reaction in any one as a positive test, we find that serum from 52 of 54 patients with malignant tumor would be positive, an accuracy of 96.3 percent, but of 193 patients without carcinoma, the serum of 117 or 60.6 percent would also give a positive result.

The use of a combination of tests may be of value in excluding a diagnosis of malignant tumor. In this group of 54 patients with cancer, the sera from only 2 or 3.7 percent gave negative reactions in all 3 tests, while in those 193 patients without a malignant neoplasm, only 28 or 14.5 percent gave positive reactions in all 3 tests. Of those 28 patients, the great majority were in the groups which are not usually a problem in differential diagnosis, *i. e.*, pregnancy, tuberculosis, acute infections, and postoperative patients, while none were in the groups with benign tumors or without apparent disease.

This type of manipulation remains of doubtful value in the attempt to diagnose the presence of malignant neoplasia in the absence of a specific reaction.

SUMMARY

Antiproteolytic reaction (A. P. L.), iodoacetate index (I. A. I.), and least coagulable protein (L. C. P.) tests were made on a series of 260 patients with a variety of diseases.

In this group of tests, the I. A. I. was most frequently positive in patients with malignant neoplasia (83.3 percent), while the A. P. L. reaction showed the highest overall accuracy (75 percent).

Using the combination of tests, if a single positive in any test be considered as evidence of malignancy, 96.3 percent of patients with malignant neoplasia gave a positive reaction, but unfortunately 60.6 percent of patients without malignant neoplasia gave a positive reaction.

If cases where all three tests are negative be considered as proof of

absence of malignant neoplasia, the error in patients with such disease would be but 3.7 percent.

If cases where all three tests are positive be considered diagnostic of malignancy, only 14.5 percent of patients without malignancy will be falsely diagnosed, and none will be in the groups with no disease or benign tumor.

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MELANOBLASTS[1] IN THE STROMA OF THE PARATHYROID GLANDS OF STRAIN C58 MICE[2]

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The parathyroid glands were examined under a dissecting microscope in a group of mice of strain C58 in which bone lesions had been induced experimentally. Although no changes were found which could be correlated with the skeletal lesions, the parathyroid glands were observed to be pigmented in many of the animals. A study of pigmentation of the parathyroid glands was then undertaken in a number of untreated mice of this and other inbred strains available at the National Cancer Institute. The nature and distribution of the pigment-containing cells are described in this report, and their significance is discussed.

MATERIALS AND METHODS

Mice of strain C58 have black coats and have been inbred for over 75 generations (Law, *1*). An account of the origin of this strain has been given by MacDowell and Richter (*2*) who used these animals in many of their studies on mouse leukemia. Strain C57 black, C57 brown, and C57 leaden (strain L) mice which are included in this investigation were derived from the same heterozygous stock as was strain C58[3] (Law, *1*).

In this study the parathyroid glands were exposed by removing the muscles overlying the trachea, the thyroid gland, and the inferior thyroid vessels. This area was then examined under the dissecting microscope at 7 and 20 times magnification. The parathyroid glands when deeply pigmented were readily found because of their dark color. Without this distinguishing feature, these minute bodies were often not clearly distinguishable from the surrounding tissues. The position of the parathyroid glands varied. One was frequently detached lying below the thyroid gland, usually on the right. A table with diagrams was arranged to indicate the position of the glands and the presence of pigment in mice of the various strains examined (table 1). The detached para-

[1] The term melanoblast is used to designate the branched pigmented cell which produces and contains melanin granules.

[2] Received for publication October 13, 1949.

[3] The mice used in this investigation were obtained from Dr. Lloyd W. Law of this Institute.

thyroid bodies and the lobe of the thyroid gland in which parathyroid tissue was recognized or suspected to be present were then removed from 30 mice of strain C58 and from 6 mice each of 5 other strains, namely C57 black, C57 brown, C57 leaden, C3H, and dba. This material was then fixed in Zenker-formol solution, embedded in paraffin, serially sectioned, and alternate slides were stained by hematoxylin and eosin and by toluidine blue. The pigmented bodies were also treated by Krajian's silver impregnation method and stained for iron by the Prussian-blue reaction. Four rats of the pigmented Long-Evans strain were treated similarly. No albino animals were included in this investigation. A number of pigmented parathyroid glands were dissected from mice of strain C58 and either squashed beneath a coverslip and examined fresh

TABLE 1.—*Pigmentation in the stroma and location of the parathyroid glands in relation to the thyroid gland and trachea* [1] [2] [3]

STRAIN MICE	NUMBER																	
C_{58}	30	9	1	2	2	2	2	1		1	2	4	1			2	1	
C_{57} BLACK	6									1				1				4
C_{57} BROWN	6				1										1	3		1
C_{57} LEADEN (L)	6				3				1						1		1	
C_3H	6																	6
dba	6									1						1		4
LONG-EVANS BLACK RATS	4							4										

[1] Diagrams indicate the presence of pigment and the position of the parathyroid glands in relation to the thyroid gland and trachea. Solid black represents a pigmented parathyroid gland.

[2] Underlined numbers in table indicate animals with pigment in one or both of the parathyroid glands.

[3] Absence of parathyroid gland in diagram indicates that it was not found on inspection.

with the phase microscope or placed in a drop of glycerine on a slide with a coverslip and allowed to clear for a few hours. Frozen sections of both pigmented and nonpigmented parathyroid glands were treated with 3,4-desoxyphenylalanine (dopa), using the method of Laidlaw (*3*), and pigmented bodies were treated with potassium permanganate and oxalic acid to determine whether bleaching occurred.

RESULTS

The table shows that one of the parathyroid glands was pigmented in about one-half of the mice of strain C58; both were pigmented in about one-third, leaving less than one-fifth in which no pigment was found.

With such variables, the present series is too small for statistical analysis. A single pigmented parathyroid gland was found in only one mouse each of strains dba and C57 black. The parathyroid glands in all other mice examined and in the four rats were not pigmented. The variation in position of the glands has been indicated by diagrams in the table. Actually this variation was greater than is shown here, for the detached body might lie closely applied to the lower part of the thyroid gland, in the neck just above the clavicle, or at any distance between these points. It was often closely applied or attached to the inferior thyroid vessels. When the parathyroid gland was in close relation to the thyroid gland, it was most often located on the superior lateral surface, but it was sometimes found at other sites on the thyroid gland, in which it was occasionally deeply embedded. This variability in position probably accounts for the frequent failure to locate nonpigmented glands without serial microscopic sections of the tissues in this region. Animals ranging in age from 1 to 18 months, and of both sexes were examined, and no difference for sex or age was demonstrated.

The pigment-containing cells in the parathyroid glands were arranged in a fine network of threadlike processes which was sharply limited to the connective tissue and capsule of the gland. No pigment accumulations were found in subcutaneous and deeper tissues of the neck unassociated with parathyroid tissue (Fig. 1).

The parathyroid parenchymal cells were readily identified in sections stained by hematoxylin and eosin, but the pigmented cells were not well visualized among the basophilic glandular cells. With toluidine blue, on the other hand, the pigmented cells stood out clearly in the connective tissue stroma, often closely applied to the vessel walls (Fig. 2). No pigment granules were found in parenchymal cells.

A remarkably clear demonstration of the pigmented cells was achieved in the glycerin preparations (Fig. 3). With high magnification the many branching processes of the cells and the nuclear space were distinctly seen (Fig. 4). The granules were dark brown and of uniform size. Under the phase microscope, they appeared discoid rather than globular.

The dopa reaction on frozen sections of the nonpigmented glands was always negative. The pigmented cells were intensely blackened after this treatment. The pigment granules were uniformly bleached by exposure to potassium permanganate and oxalic acid. In the paraffin sections they were intensely blackened after treatment by the Krajian silver technique and were negative to the Prussian blue reaction. Because of these observations, the pigmented cells were considered to be melanoblasts.

DISCUSSION

Only a few previous reports on the occurrence of pigmented cells in the parathyroid stroma have been found. Addison and Fraser (*4*) reported that branching cells with pigment granules (which they termed melanophores) were found rarely in the parathyroid glands of wild Norway rats.

In about one-half of the same group of animals, pigmentation was found in the pars intermedia of the hypophysis. The hypophyseal pigmentation was often extreme, due to the aggregation of many pigmented cells. These cells might sometimes be present in the parathyroid stroma and absent in the pars intermedia, and the pigmentation sometimes occurred in only one of the parathyroid bodies. It was remarked that the cells were closely associated with the vessels of the stroma. Twenty wild gray mice were examined, and no pigment was found either in the hypophysis or the parathyroid gland. These authors remarked that no reference to melanotic pigmentation in the parathyroid glands of other mammals had been found. However, in a paper published in 1895, Schaper (*5*) described cells in the stroma of the parathyroid glands in sheep which contained a considerable amount of fine granular pigment. These cells had numerous protoplasmic processes and were especially seen in the neighborhood of capillaries and small arteries.

More recently Foster (*6*) has made a cytological study of the parathyroid glands from a number of noninbred mice. While he gave no detailed description of melanoblasts, he stated, "In a few of the younger glands, irregularly-shaped, elongated cells containing yellowish-brown pigment granules were occasionally seen associated with the connective tissue around the larger blood vessels."

No description of melanoblasts in the parathyroid stroma of man has been found in recent reference books and papers on the parathyroid gland [Maximow and Bloom (*7*), Cowdry (*8*), Grollman (*9*), Marine (*10*), Castleman and Mallory (*11*)]. That such cells may be present, however, is suggested by Gilmour (*12*), who described in the perivascular connective tissue of the parathyroid gland, spindle cells sometimes as much as 6 by 60 μ in size which often contained much finely particulate yellow-brown pigment. He interpreted these cells as adipose tissue cells from which the fat had disappeared, although he was at a loss to explain that if the pigment was the same as that seen in other adipose cells, it had certainly increased in amount and become more finely particulate and darker. Erdheim (*13*) in 1907 recognized pigment-filled cells in the parathyroid stroma in human beings. He found these cells in close association with the great vessels, particularly the arteries. The cells were described as elongated, with the long axis generally parallel to the direction of the vessel and containing small, spherical pigmented granules in the cytoplasm, with a clear nuclear space. In Muller-formol preparations, the pigment was a light yellow brown, but when treated with osmic acid, the granules became dark brown. The iron reaction was negative. Erdheim remarked that these cells could be compared with similar cells in the leptomeninges and that they were found at both sites more frequently in older individuals. An exact age correlation, however, could not be drawn since much more pigment was found in one subject aged 50 than in one aged 77. He noted also that the numbers of pigment cells varied in the four parathyroid bodies, sometimes being present in one

only. He emphasized that these pigmented cells were readily distinguishable from pathologic cells with ingested blood pigments, although he reported that he had not found pigmented cells in any individual under 50 years old, which would lead one to believe that he regarded them as an aging phenomenon. Haberfeld (*14*) likewise saw such cells in the parathyroid stroma of a 55-year-old man, and he described them as being situated around the larger vessels. He agreed with Erdheim in considering them a normal condition, although he thought they were found only in older people. They did not give an iron reaction and were not associated with the hemorrhages which he described in many of the glands examined.

Taking heed of these reports by Gilmour, by Erdheim, and by Haberfeld, and considering the situation in mice, an investigation of the parathyroid glands in man using glycerine-cleared, nonstained tissue might disclose melanoblasts in occasional individuals. A search among members of the dark-skinned races in which the meninges are also pigmented might be especially rewarding.

No explanation for the presence of pigmented cells in the connective tissue of the parathyroid glands of mice can be offered. It seems highly improbable that a functional relationship exists, since pigmented cells are not regularly found, even in strain C58 mice. It may be conjectured that during embryonic development, the parathyroid anlage was closely associated with melanoblastic tissue which became incorporated with it, and these two elements moved together to the final location in postnatal life. To prove such a hypothesis would require a study of early embryonic stages. Rawles (*15*) made a study of the origin of pigment cells in the mouse and concluded that only those tissues containing presumptive neural crest, histologically recognizable neural crest, or cells migrating from the neural crest could produce "melanophores." (She defines as melanophores the migratory, pigment-forming cells, from which the cells designated here as melanoblasts are formed.)

It may be relevant to this discussion that pigment has often been observed in the meninges at the base of the brain in C58 mice and microscopically in the valves of the heart. No accurate record has been kept, however, as to the frequency of this pigmentation or as to whether it is correlated with the pigmentation of the parathyroid glands. Pigment has not been observed in the thymus or thyroid glands, which are reported to have an origin similar to that of the parathyroid glands.

The pigmentation of the parathyroid glands of strain C58 mice may be of practical value if parathyroidectomy is contemplated in mice, for when pigmented, the bodies are readily identified, and they can then be removed. Such pigmented glands also afford a beautiful demonstration of dendritic melanoblasts and can be utilized whenever such cells are desired for morphological study.

It seems highly improbable that there is an association between the presence of leukemia and the pigmentation of the parathyroid-gland

stroma. Continued observations on C58 mice, however, will determine this possibility.

SUMMARY

Branching pigmented cells identified as dendritic melanoblats have been found in the stroma of the parathyroid bodies in mice of certain inbred strains. They were frequently found in mice of strain C58 but were also rarely found in strains dba and C57 black. Such cells were not found in four rats with black coats of the Long-Evans strain.

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PLATE

PLATE 107

FIGURE 1.—Pigmented parathyroid bodies in situ. On the right in the photograph the body is seen attached to the thyroid gland. On the left it lies below the thyroid gland along the course of the inferior thyroid vessels. × 8.

FIGURE 2.—Melanoblasts in the stroma of the parathyroid gland. Paraffin section. Toluidine blue stain. × 400.

FIGURE 3.—Parathyroid body removed with surrounding thyroid tissue and cleared in glycerine to demonstrate melanoblasts. × 200.

FIGURE 4.—Dendritic melanoblasts from stroma of parathyroid gland. Nuclei can be indistinctly seen as spaces free of granules. Glycerine cleared, unstained preparation. × 1,200.

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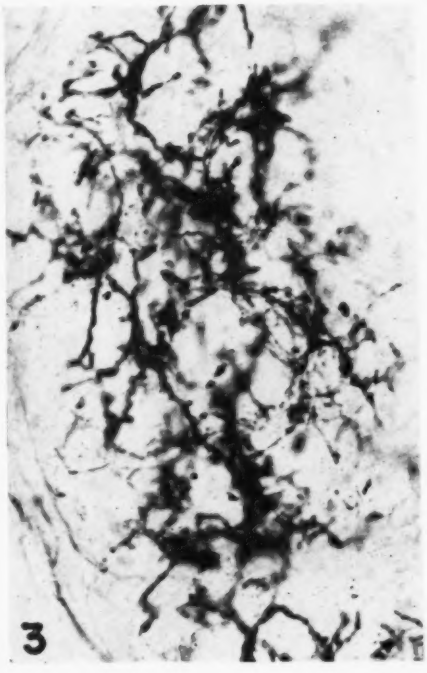

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THE NATURE OF RADIATION INJURY TO AMPHIBIAN CELL NUCLEI [1]

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The major problem dealt with in this paper concerns the physiological mechanism by which cell nuclei are damaged by ionizing radiations. It is an attempt to give specific answers to the following problems: (1) What are the destructive effects of radiation in the nucleus? (2) How are these brought about? The present incomplete status of roentgenological pathology is clearly and concisely set forth in Bloom's recent monograph on the histopathology of irradiation (*1*). Zirkle (*2*) has analyzed certain important theoretical chemical relationships between physical and biological events in irradiated cells. Other excellent and extensive reviews [2] have been published by Giese (*3*), Lea (*4*), Hevesy (*5*), Warren (*6*), and others. In referring to the writer's earlier work, Giese states (p. 269): "Duryee's results on frog eggs are used to support the contention that the cytoplasm is mainly affected, although the author himself made no such claims." It should be added that the indirect action of radiation on the nucleus was evident from the early experiments (*7*, *8*) although no mechanism was then postulated.

The work of the author referred to was reported in two preliminary notes. Although many additional experiments have been undertaken since then, only those dealing with roentgen radiation on various species of amphibian eggs are included here.

In analyzing the problem, certain factors best summarized in Giese's review, should be kept in mind. He carefully points out that "two theories of the mechanism of action of ionizing radiations have arisen—that of direct action (target theory) and that of indirect effect by ionization of water" and that "specific effects such as gene mutations and effects of chromosomes are thought to be due to direct hits, while less specific effects, such as delay in cleavage, may be due to indirect as well as to direct action of the radiations." Recently Barron (*10*) offered a

[1] Received for publication October 18, 1949.

[2] The most extensive bibliography of radiation effects is that published by the United Nations Atomic Energy Commission in 1948 (*9*).

better hypothesis for indirect action based on formation of stable organic peroxides.

Particular attention should be given to the useful data and conclusions of Patt and Swift (*11*) who studied longevity of frogs radiated at different temperatures with dosages directly comparable to those reported here. They concluded that "primary reactions occurring during exposure . . . are independent of body temperature and are apparently physical or photochemical,[3] while . . . secondary reactions are temperature sensitive."

What has been most needed is a new and direct experimental approach inside the cell. In this paper, new, roughly quantitative criteria of damage to the nucleus are presented. Techniques developed by the writer (*13*) for isolating single cells, individual nuclei, chromosomes, nucleoli, and measured volumes of cytoplasm from ovarian eggs have made possible irradiations of specific cell parts, either singly, in pairs, or in other combinations. Effects on isolated parts were checked by concomitant observations on the whole transparent cells and by irradiations of the entire animal. Separation of temperature insensitive reactions from those with a high temperature coefficient has been sought. In extension of the conclusions of Patt and Swift, it will be shown that intracellular radiation damage is a threefold phenomenon consisting of: (1) primary, physical, or radiochemical changes in the cytoplasm with low temperature coefficients, and (2) secondary chemical and metabolic processes which are highly temperature sensitive and which accumulate or result in an unidentified protoplasmic nuclear toxin, and (3) transmission processes into the nucleus. The last has been demonstrated by the experiment of causing pyknosis with irradiated cytoplasm injected against the nuclear membrane.

As parameters, we have radiation procedures, isolation techniques, cellular behavior, micromanipulation methods, and optical systems. Emphasis is on new cytological data and new approaches to the radiation problem, rather than debate with previous theories.

The conclusion that nuclear injuries (such as chromosome fragmentation, nucleolar damage, spindle substance change, and chromomere loop dissolution) can, to a large degree, be under cytoplasmic control is in accord with the conclusions of Stone (*14*), Wyss (*15*), and Barron (*10*) that radiation effects may be secondary and indirect. This finding does not necessarily conflict with the target hypothesis, as developed by Lea (*4*), Sax and Swanson (*16*), Catcheside (*17*), and others. It is clear that our cytological techniques do not now have direct bearing on the kind of abnormalities studied by the techniques of genetics. However, experimental cytology has immediate value in the correlation of data from many sources. Here are brought together effects on whole animals, isolated organ systems, isolated cells, and single cell parts in physiological media.

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[3] The word photochemical as used above refers to the instantaneous events occurring during ionization by absorbed quanta of radiant energy. Since it now appears likely that the mechanism of ultraviolet effects is basically different from X-radiation effects, the term radiochemical seems preferable and will be used hereafter to refer to the instantaneous physical effects involved in either X-, beta, or gamma ray absorption (*Cf.* Rossi's discussion (*18*) of the nature of ionizing radiations).

A pertinent section includes experiments on the transfer of radiated cytoplasm to nonirradiated cells. Although many of the eggs have been studied in cross-section, it should be stressed that, unlike most data in the literature, this cytological analysis is derived entirely from the study of fresh, unfixed material.

MATERIALS AND METHODS

Irradiations were carried out at the Marine Biological Laboratory and at the National Cancer Institute.[4] In both institutions, the X-ray generators are of the same design: two G. E. Coolidge tubes were mounted above each other and operated from the same transformer so that one alteration of the high voltage current passed through one tube and the other alteration through the second. At the M. B. L. the tubes were operated at from 182 to 194 kv. and 25 ma., through each tube. At the National Cancer Institute the tube voltage was 186 kv. and the current 20 ma. No filters were employed. Target distance from center of irradiated area was 35 cm. for whole animals, 9.5 and 12.7 cm. in the case of eggs and nuclei. Irradiation rates ranged at the M. B. L. from 7,600 r/minute, in a few of the early experiments, to 6,100 r/minute on the isolated cells. Whole animals were irradiated at 4 different dosage rates: 450-500 r/minute (N. C. I.) and 2,800-6,100 r/minute (M. B. L.). No significant differences were observed between effects of the different rates. Total doses varied in graded steps from 500 r to 100,000 r in a few cases. The great majority of experiments was done at the 30,000–60,000 r level with individual eggs and at the 300 r level with whole animals.

Whole animals were irradiated in batches of 6–12 individuals in order to make the data more exactly comparable. It was found convenient to wrap them lightly in cellophane packages fastened with Scotch tape. In this manner, they were kept entirely within the radiation field. Ovaries and single eggs were exposed in transparent plastic cups of appropriate size. Isolated nuclei, obtained through the technique described earlier (Duryee, *13*, *18*) were placed in special lucite holders consisting of three 3-mm. diameter holes 2 mm. deep, bored in 4 mm. thick strips. Each well contained approximately 4 mm^3. of N-medium (NaCl 0.5 percent KCl 0.02 percent). Exposure of untreated isolated nuclei to a cytoplasmic brei was made by grinding up the contents of 3 eggs (from which nuclei had been previously removed) in 4 mm^3. of N-medium. Membranes were carefully abstracted. In the lucite holders described, this preparation was irradiated. Nuclei were exposed to the brei for approximately 1 hour. After irradiation, eggs were either examined immediately or the animals were placed in jars cooled with running tap water at 20° –24° C. In other cases, irradiated animals were quickly placed (3–5 minutes later) in an ice-

[4] Special thanks are due to Dr. E. Lorenz of the National Cancer Institute and to Dr. G. Failla of the Marine Biological Laboratory for providing X-ray facilities. The efficient cooperation of the Messrs. Little and Hyde of the Marine Biological Laboratory and H. Meyer of the National Cancer Institute in carrying out the radiations is gratefully acknowledged.

box running at 4–6° C. On removal from the cold, animals were returned to warmer containers, adjacent to those of the controls.

Microinjection technique consisted of withdrawing cytoplasm from transparent ovarian eggs mounted in amphibian Ringer pH 7.8 and injecting the material immediately (2 minutes) into other normal eggs against the nuclear membrane. The micropipettes were made of pyrex drawn in a Livingstone needle-puller. They were mounted on a Chambers micromanipulator fitted with a screw-operated syringe. Donor and host eggs were mounted separately in two small hanging drops in a moist chamber, after being carefully cut from the ovary with special iridectomy scissors. By trimming under the binocular scope, all damaged cells were first removed, and the small egg masses were then oriented in the hanging drops so that the ovarian wall was uppermost to facilitate penetration by the pipette. A fresh pipette was used for every experiment so that each donor cell was punctured by a very sharp glass tip. Each egg was held steady with a microneedle by pressing the theca cells against the coverslip. Pipette tips were broken through the top of the egg against the coverslip and quickly lowered into the cytoplasm, where vortical currents and granular movements evidenced good withdrawal. Cellular shrinkage was then measured. Injection followed immediately, and the mass of cytoplasmic material could be seen mixing with the host cytoplasm and displacing and flattening the nucleus. Volumes of injected material mass were calculated roughly from diameters measured with an ocular micrometer. Photomicrographs were routinely taken during these operations.

Results in this paper are based on a study of living ovocytes from 179 salamanders and 123 frogs. In the former group the animals were of two species: *Triturus pyrrhogaster Boie*, obtained directly from Japan,[5] and *Triturus viridescens Rafinesque*, collected locally. Most of the frogs used were *Rana pipiens Schreber*, with some experiments noted below on *Rana catesbiana Shaw* and *Rana clamitans Latreille*. No significant differences were found between species of the same genus. In general, urodeles were observed to have higher radiation tolerances than anurans as measured by longevity. Also, isolated cells were markedly less radiosensitive than whole animals. In one experiment, localized shielding of the ovarian area of a salamander was provided by surrounding the posterior trunk region with a lead wrapping one-fourth inch thick.

RESULTS

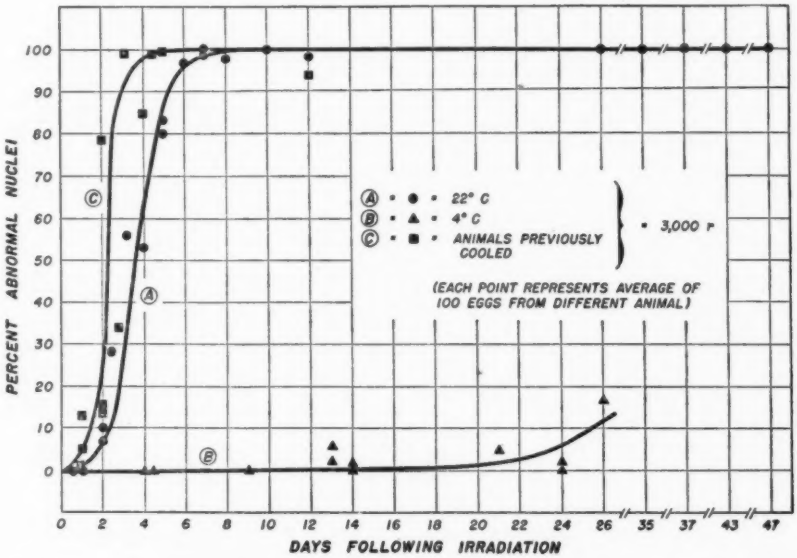
The data from these experiments are presented under three separate headings. Part 1 includes the experiments deriving from the irradiation of whole animals (salamanders). This part includes effects of low temperatures postirradiation in delaying nuclear injury. Part 2 includes irradiation effects on isolated ovaries, on single eggs, on isolated nuclei and chromosomes, and on fresh concentrated cytoplasmic brei. Part 3

[5] Thanks are also due to Col. R. E. Tigert of the 406th Medical General Laboratory and Dr. A. Stull of the Surgeon General's Office for arranging these shipments.

consists of data on microinjections of normal ovarian eggs with irradiated and normal cytoplasmic material. Only the data from Part 2 has been reported in preliminary notes (*7*, *8*, *19*).

1. Nuclear Injury Resulting From Irradiation of Whole Animals

As has long been known, germ cells are classed among those most sensitive to radiation. In this group of 80 salamanders, graded irradiation doses from 1,000 r to 10,000 r were employed in order to analyze the mechanism of injury. Results are summarized in tables 1 and 2. The tables demonstrate that: (1) nuclear injury is increased with increasing dose, (2) the percentage of visibly affected small cells is a function of the number of days following irradiation, and (3) nuclear injuries may be postponed by exposure to low temperature, although no recovery takes place. The striking type of pyknosis resulting from radiation found in the small transparent eggs (200–300μ in diameter) is illustrated in Figures 1, 2, 3, 4, and 5. Details will be explained later. Attention first is called to the various dosages. At the critical 3,000 r range, nuclear damage is especially interesting. When these figures are plotted against time, the curve *A* as shown in text-figure 1 is obtained. Points on the curve were obtained from cell counts in randomly selected fields. Ap-



Text-figure 1.—Relation between percentages of abnormal nuclei in ovarian eggs and the number of days following irradiation. Curve *A* indicates rate of nuclear injury appearing at room temperature. Curve *B* shows the delay of visible injury when animals are kept on ice. Curve *C* shows the accelerated rate of injury appearance following any period of cooling.

proximately a hundred cells were counted from each individual. With few exceptions, data from comparable individuals and species varied only ±5 percent.

TABLE 1.—*Summary of proportional damage in ovaries x-rayed in vivo, and maintained at warm temperatures (22°–24° C.). Small transparent eggs* (Tr. viridescens, Tr. pyrrhogaster)

Radiation dosages	Animals	Number of days postirradiation	Average percent of nuclear damage in transparent ovarian eggs
	Number		
1,000 r	1	26	0
Total	1		
2,000 r	1	5	24
	1	7	85
Total	2		
3,000 r	2	1	0
	4	2	28
	3	3	58
	3	4	74
	2	5	81
	1	6	97
	5	7	91
	1	8	65
	1	10	100
	1	12	98
	1	26	100
	1	35	100
	1	37	100
	1	43	100
	1	47	100
Total	28		
4,000 r	1	1	0
	1	4	90
	1	34	100
	1	44	100
Total	4		
5,000 r	1	2	4
	1	11	100
	1	11	100
	1	22	99
	1	26	100
Total	5		
10,000 r	1	2	17
	1	2½	18
	2	4	94
	2	5	91
	1	20	100
	1	22	97
Total	8		

TABLE 2.—*Summary of proportional damage in ovaries x-rayed in vivo, followed by exposure to cold and warm periods. Small transparent eggs.* (Tr. viridescens, Tr. pyrrhogaster)

Radiation dosages	Animals	Days postirradiation		Average percent of nuclear damage in transparent ovarian eggs
		4° C.	22-24° C.	
	Number	*Number*	*Number*	
3,000 r	2	4	0	0
	1	9	0	0
	2	13	0	5
	1	14	0	2
	1	21	0	48
	1	21	0	5
	1	24	0	2
	1	26	0	17
	1	5	1	1
	1	7	1	5
	1	10	1	13
	1	5	2	79
	1	7	2	14
	1	9	2	14
	1	7	3	35
	1	21	3	100
	1	10	4	85
	1	13	4	20
	2	13	5	100
Total	21			
5,000 r	1	2	0	0
	3	12	0	0
	2	12	12	95
Total	6			
10,000 r	1	5	0	0
	1	5	0	1
	1	12	0	0
	1	12	7	100
Total	4			

From a study of this curve, it was found that there is a lag period of from 1 to 2 days before extensive pyknosis appears. This is followed by a 3-day period when nuclear injury is appearing rapidly and an end period wherein a diminishing number of small eggs show relative resistance until, by the seventh day, over 98 percent of the eggs are dead or moribund. After a dose of 3,000 r at room temperature, it should be emphasized that no recovery has been observed and that, although animals are active and feed well, the entire germinal tissue is destroyed by the 16th day or earlier. Only black, granular masses of the gradually disappearing eggs can be found among few remaining engorged blood vessels and disintegrating stroma. Of paramount importance is the contrasting fact that nuclei of the larger, more mature [6] eggs (1 mm.) are not visibly

[6] Accurate stages of ovarian development are important in cytological and physiological comparisons. The writer has found it convenient to class the smallest eggs (100–150μ) as Stage 1, those from 150–200 μ as Stage 2, those from 200–500 μ as Stage 3, yellowish brown eggs of 500–750 μ as Stage 4, "half size" gray brown eggs 750–850 μ in Stage 5. Stage 6 includes all the larger eggs up to 1,800 μ in diameter. Experiments reported in this paper deal chiefly with Stage 3 and Stage 6 eggs.

affected in these intervals by comparable irradiation doses. It was noted, however, that all eggs in two 4,000 r treated individuals had been destroyed at the time of their respective deaths after 34 and 44 days of postirradiation.

Salamanders were found to survive roentgen irradiation better than frogs. Following 1,000 r some individuals died after 26 days at room temperature, although their eggs showed no visible injury at that time. With a dose of 3,000 r, individuals can live at least 35 days, and probably longer, although the ratio of injured to normal small eggs approached 100 percent by the seventh day. The longest survivors happened to be 2 females given 4,000 r that lived 34 and 44 days respectively. It was found that 2 animals irradiated with 10,000 r died on the twentieth and twenty-second days. Untreated controls lived under the same conditions of water, temperature, and feeding for many months. In contrast to the more resistant salamanders, frogs lived only 11 days after 3,000 r and 7 days after 10,000 r. While these survival periods might be expected to vary further in a more greatly extended series of tests, they do, nevertheless, give a clear picture of the radiation susceptibility of *Triturus pyrrhogaster* as compared with female *Rana pipiens*.

EFFECT OF LOW TEMPERATURES

A series of experiments was undertaken to find out whether radiation injury to the egg nuclei could be prevented by lowering the temperature within a physiological range. From each batch given a certain dose, a portion was immediately transferred to the ice box maintained at 4°–6° C. At appropriate intervals, animals were removed and returned to room temperatures.

Two striking facts are evident from the data in table 2 and from curves *B* and *C* in text-figure 1. One is that pyknotic changes in the small transparent eggs can be temporarily arrested, but not for more than about 2 weeks (Curve *B*, text-figure 1). The other is that nuclear injury appears progressively, at a comparable rate (Curve *C*, text-fig. 1) to that shown previously in Curve *A* and in table 1, according to the number of days of rewarming. As an example of the first conclusion, animal x_3 irradiated with 3,000 r and kept 13 days in the cold was allowed to warm to room temperature (22° C.) for 4 hours. Of 262 small transparent eggs examined, 4 showed dense central nuclear bodies and 10 had centrally clumped chromosomes, an average of 5 percent abnormal. Nuclei of the other 248 cells appeared entirely normal. This may be compared with another *Triturus viridescens* 12 days postirradiation but maintained constantly at the warmer temperature of 20.5° C. In this female, only 2 of 85 eggs were still normal, and the abnormality figure was 98 percent. In further contrast, low temperature following a higher dose of 5,000 r was able to prevent visible nuclear damage in 3 different animals as late as 12 days after the treatment. It should be remarked, however, that considerably longer periods in the cold, *i. e.*, 21 days, allowed up to 48 percent of the eggs to show injury.

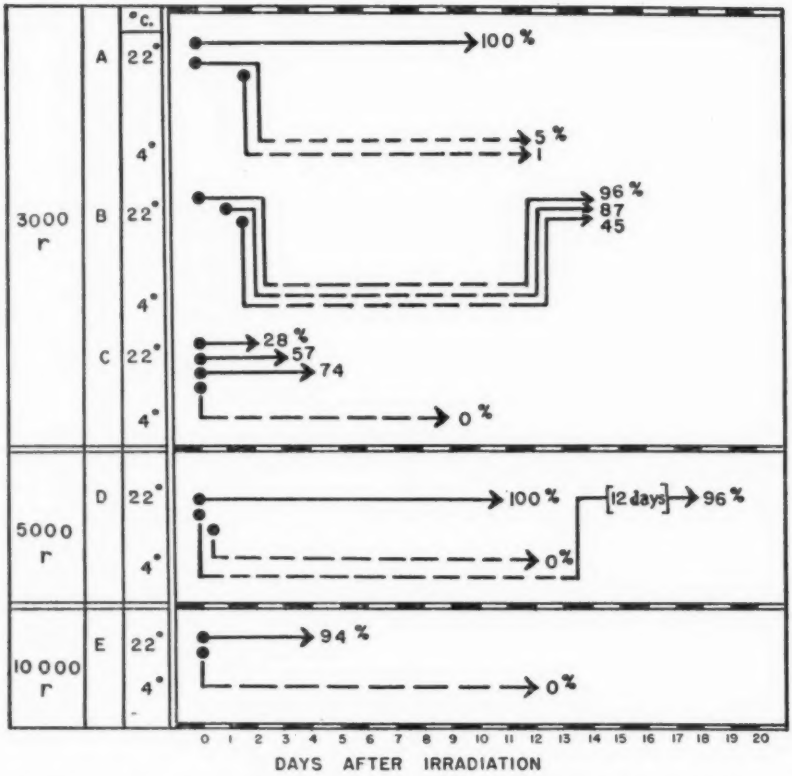
Once it had been established that nuclear injury is not prevented but only delayed by low temperature, the question arose whether the toxic mechanism was metabolic in character. Three experimental approaches to this problem were possible. They are described in the following paragraphs.

One was to see if higher temperatures accelerated the rate of nuclear disintegration. A small series of animals were maintained at 27° C. ± 0.5° for 3 days. At autopsy these had an average of 84 percent damaged nuclei, as compared with 53 percent for those kept at 22° C. postirradiation. This finding strongly supports the metabolic hypothesis.

Another approach was to analyze the characteristics of the curves in text-figure 1. It is evident that the shapes of both Curves *A* and *C* are quite similar but that Curve *C* is displaced toward the left. This indicates that approximately 50 percent of the nuclei showed pyknosis by the second day after rewarming instead of on the third day as in Curve *A*. This fact plus the observation that longer exposures to cold (over 20 days) allowed some damage to appear (0–20 percent) constitutes evidence that the slow metabolic phase of the injury process was still capable of operating and had not been entirely suppressed by ice box temperature.

A third experimental approach was to divide the periods of warmth and cold into different fractions and to vary the sequence of the treatment. This series is shown diagrammatically in text-figure 2. It shows in *A* that a 2-day period at the higher temperature before cooling does not alter the predictable injury ratio. Figure 2, *B* also demonstrates that for animals given 0, 1, and 2 days of higher temperatures before cooling and a following 2-day period of warmth after an equalized 10-day period in the cold, there is a higher percentage of injured nuclei than would be quantitatively predicted for corresponding 2-, 3-, or 4-day periods of warmth alone. This strongly reinforces the conclusion that a small amount of protoplasmic nuclear toxin can be produced even in the cold. Figure 2, *C* illustrates four typical experiments from straight runs. In Figure 2, *D*, it is of interest that at a still higher dose, few or no cells are visibly damaged by 12 days, as long as they are kept cold. This also shows that the low temperature does not prevent the primary radiochemical action of X-rays; it merely delays the cellular factors of secondary damage. That this retarding action is, however, very strong is brought out by diagram E showing that a dose of 10,000 r (lethal at room temperature) can be controlled for many days.

Another question which deserved attention was whether these cytological effects were due to local penetration by the X-rays of the eggs themselves or whether they were due to generalized systemic effects or so called "necro-humors." This was tested in a single experiment at the 3,000-r level in which a 90-mm. animal was shielded for 16 mm. around the posterior trunk region by a lead girdle 6 mm. thick. The results showed that only 0.8 percent of the 170 cells had developed nuclear injury. Since the observation was made on the seventh day postirradiation, when con-



TEXT-FIGURE 2.—Percentages of nuclear injury following irradiation and maintenance of salamanders at different temperatures. Percentages refer to the ratio of abnormal nuclei to normal ones. Heavy circles indicate day on which animals were X-rayed.

trol experiments indicated nearly 100 percent destruction, it was concluded that (1) the shielding of the ovaries by the lead was nearly complete and (2) X-ray injuries resulted, not from indirect systemic effects, but from localized events in the cells themselves. This conclusion is strongly upheld by experiments with β-radiation to be reported in a separate paper.

CYTOLOGICAL EFFECTS *IN VIVO*

Earlier in this paper the desirability was pointed out in both radiology and pathology for more intracellular information. Examination of Figure 2 shows a highly magnified view of the nuclear interior of a transparent egg mounted in buffered Ringer solution. It was taken from an animal given 3,000 r, kept at 4° C. for 7 days and at 22° C. for 2 days. In comparison, Figure 3 shows the nuclear interior of a cell similarly irradiated, but kept 2 days at 22° C. only. The early stages of pyknosis have pro-

gressed slightly more in Figure 2 than in Figure 3. Two nearly normal cells may be seen in Figure 1 for comparison.

Beginning radiation damage is as follows: (1) Prophase chromosomes normally transparent and invisible become shorter, thicker, darker, and beaded. (2) In young ovocytes the nascent chromomere lateral loops swell and disintegrate. (3) Nucleoli along the inner surface of the nuclear membrane evert their contents into the cytoplasm, and their residual shells swell and disintegrate. (4) The central nuclear ground substance (karyosomal substance or spindle substance), which the writer has termed the *Chromosome Frame*, changes from a gel to a sol, thereby allowing the chromosomes to tangle, mat, and clump. (5) Later radiation damage with advanced pyknosis consists of formation of a clumped central nuclear body (C. N. B.). Progressive stages are illustrated in Figures 3, 4, and 5. These processes involve a fundamental phase separation of the nuclear colloid, with a coating of the chromosomes (Figures 3 and 4) under the influence of the condensing phase's *own* specific gravity. It should be noted in the photographs that the chromonemata retain their linearity only during the early stages of this process. The intricate nature of cellular death changes are not easy to understand from plane photographs alone. Cells should preferably be studied microscopically in three-dimensional relationships and in normal time sequences.

It is allowable to conclude that the above five stages of pyknosis apply in partial measure to somatic cells likewise, since most of these events may be recognized in the mesothelial theca cell nuclei that surround each egg. One of the advantages of living amphibian material is that both the large egg nuclei, with perfect detail, and the smaller somatic cell nuclei, with less detail, can be studied simultaneously in the same microscopic field, as illustrated in Figure 2.

From an analysis of the many hundreds of eggs in this series of whole-animal-irradiation experiments, the following general conclusions have been drawn:

(1) Visible nuclear damage to ovarian eggs followed 3,000 r doses of irradiation after a 1-day lag period.

(2) Degree of damage could be expressed by the proportional numbers of cells with injured nuclei.

(3) Higher doses produced more damage in shorter periods than did lower doses.

(4) Nuclear injury could be delayed many days by low temperatures (4°–6° C.) but not prevented.

(5) Higher temperatures (25°–27° C.) accelerated production of toxic effects.

(6) Accelerated production of nuclear injury appeared on termination of the cooling period.

(7) Primary nuclear injury was preventable by local shielding with lead. Therefore, results were the outcome of direct radiation effects within the ovary and not from general systemic changes.

(8) Conclusions could be applied also to somatic nuclei and not specifically to reproductive ones.

2. Nuclear Injury Resulting from the Irradiation of Single Cells and Cell Parts

At the Marine Biological Laboratory general meeting in 1939, the writer discussed the question: Does the action of X-rays on the nucleus depend on the cytoplasm? (*8*). This section presents in detail the data on Anurans presented then, together with those from many subsequent experiments on eggs of the Japanese salamander, *Triturus pyrrhogaster*. It was considered important to run experiments in pairs, one ovary being used for control and the other for experimentation. Treated and untreated ova and the nuclei were isolated in the same solutions and mounted in adjacent drops on the same slide for comparative cytological observation. After preliminary experiments had established criteria and normal degrees of variation, the repeatability of results at a bracket of the 0, 1,000, 30,000, and 50,000 r doses could be diagnosed from material set up as unknowns and code-labeled by another person. As mentioned earlier, individual cells were studied by excising 5–10 of the half-size eggs from the ovarian wall in Amphibian Ringer, buffered to pH 7.6. In a typical experiment, samples of eggs were removed from the irradiation field every 1 minute and 34 seconds. In this manner since the dose rate was approximately 6,100 r/minute: the first set received 10,000 r, the second set 20,000 r, the third 30,000 r, and so on through 70,000. A control set was removed from the dish before the radiation started.

Isolation of nuclei followed within 3–15 minutes. Ova were gently punctured with fine forceps and the cytoplasm and yolk squeezed into unbuffered calcium-free Ringer solution (N-medium). Here the nuclei were identified, quickly washed clean of cytoplasm (an essential step), and mounted in groups of 3–10 in 4 mm^3. N-medium on glass slides under coverslips supported by plastic clay feet. When dealing with isolated cell components, irradiation was accomplished either by exposing the slides directly or by placing several nuclei in small lucite holders each containing 0.04 cc. of N-medium. The holders were then placed between the two X-ray tubes.

Units of cytoplasmic brei were prepared by first removing the nuclei individually from 3 eggs and pressing out the cytoplasm in 0.04 cc. of N-medium. Egg membranes were removed with fine needles, and the remaining mixture was ground with a glass rod in a lucite cup. Consequently, subsequent effects of this mixture on immersed, isolated nuclei from normal cells could not be attributed either to injured cell membranes or to disintegration products of the nuclei themselves.

Illustrating the experimental approach and the type of data obtainable, two typical protocols are shown in table 3. Photomicrographs were taken of the nuclear interior to record cytological detail. Standardized criteria of nuclear injury were adopted with corresponding symbols, as set forth

TABLE 3.—*Two typical protocols of irradiation experiments of isolated amphibian ova*

A. Fresh ovary: *Rana pipiens* placed in 20 cc. of amphibian Ringer, pH 7.8. Radiated in stender dish at rate of 7,600 r/minute. Control also placed in A. R. Nuclei isolated 3–15 minutes later

Radiation conditions	Ground substance	Chromosome frame	Nucleoli	Chromosome fragmentation	Loop structure	Nuclei examined
						Number
Control	Clear	Normal	Normal	None	Big loops on all chromosomes. Average 15μ.	9
NOTE.—Condition checked 11 hours later; no change from above in 8 nuclei.						
500 r	Clear	Normal gel	Normal	None	Big loops on all chr	4
30,000 r	Clear	Some swelling	Swollen, everting	Small	Only 1 complete loop found.	5
50,000 r	Clear	Partial sol	Swollen, fragmented. Average 25μ.	Many small fragments; no complete chromosomes.	No loops, only thin chr. segments left.	6

B. Fresh ovary: *Triturus pyrrhogaster* opened 9 a. m. in A. R., pH 7.6; placed in celluloid dish, 5 cc. A. R. Irradiation rate: 6,120 r/minute. Nuclei isolated 5–15 minutes later

Control	Clear	Normal gel	Normal 8–14μ	None	Loops numerous on all. Average 21μ.	3
20,000 r	Clear	Expanded	Swollen, vacuolated	Many fragments	Beaded matrix only	4
50,000 r	Clear	Sol	Heavy fusion, masses 40–90μ.	Many fragments—50μ or less.	All loops gone	5

TABLE 4—*Differential effects of X-rays on separate nuclear components of isolated amphibian eggs*

A

Radiation dose (r)	Animals	Chromosome fragmentation [1]	Lateral loops dissolved [2]	Spindle substance change [3]	Nucleolar damage [4]
	Number				
500 r	5	0	L	0	−
1, 000 r	10	0	L−	0	±
2, 000 r	4	0	L−	0	+
5, 000 r	4	0	−	±	+
10, 000 r	4	±	−	±	++
20, 000 r	5	±	−	±	++
30, 000 r	15	+	−	±	++
40, 000 r	8	++	−	±	++
50, 000 r	29	++	− −	+	+++
60, 000 r	10	+++	− −	+	+++
70, 000 r	1	+++	− −	++	+++
100, 000 r	1	+++	− −	++	+++
Total	96				

B

Cytoplasmic brei: 50,000 r	8	0	−	+	++
Cytoplasmic brei-normal	3	0	L	+	−

C

Isolated nuclei: 30,000 r	3	0	L	0	−
60, 000 r	14	0	L	+	+

[1] Fragmentation of chromosomes:
- Chromonemata longer than 200μ ..... =0
- Chromonemata shorter than 200μ ..... =±
- Chromonemata shorter than 200μ ..... =+
  (Separate or attached allele fragments of 30μ or more.)
- Chromonemata shorter than 200μ ..... =++
  (Chromosome cylinders swollen to 2μ in diameter.)

[2] Dissolution of chromomere lateral loops:
- Chromomere loops present (average 20μ) ..... =L
  (Evenly distributed along the chromonema.)
- Absence of loops over small segments of chromonemata ..... =L−
- Only few loops (average 7μ) remaining attached (dissolving loop fragments adjacent to chromosomes) ..... =−
- No loops remaining (granular fragments of loops=1μ) ..... =− −

[3] Colloidal change in chromosome suspension frame (spindle substance):
- Normal geometry of chromosomes: centrally located, not touching one another (nucleoplasm gelled) ..... =0
- Displacement of few chromosome pairs ..... =±
- Only few chromosomes remaining in central position (partial solvation) ..... =+
- All chromosomes dropped to bottom of nuclear membrane (central gel to sol) ..... =++

[4] Nucleolar damage:
- Normal appearance ..... =−
- Some nucleolar vacuoles enlarged ..... =±
- Many nucleolar vacuoles enlarged ..... =+
- Nucleolar vacuoles everted, surfaces wrinkled ..... =++
- Nucleolar vacuoles bursting, swollen, nucleoli fusing ..... =+++

in table 4. Examples are given in Figures 6, 7, 8, 9, and 12. It should again be emphasized that actual observation under the microscope reveals all of the three-dimensional geometry of a nucleus impossible to convey fully in a plane photograph and not accurately demonstrable by fixing and sectioning techniques. Although sections were made of both male and female germ cells and Feulgen staining was done, no new or different conclusions were drawn. The main character and relatively slight degree of variability of the results are summarized in table 4*A*.

From this table it is apparent that the different components of the nucleus were not equally radiosensitive. Under the conditions of the experiment, no visible injury occurred with 500 r. It is of course possible, and even likely, that new techniques will reveal more delicate injuries. Nevertheless, these data first showed visible irradiation effects after 1,000 r, which was slightly lower than the comparable 2,000 r dose at which injury appeared in the whole animal experiments (*cf.* table 1).

Although the attempt was made to obtain rough quantitative data, conclusions should allow for approximately 10 percent individual variation. On this basis, the following conclusions from short-term experiments on isolated frog eggs are inescapable:

(1) At least 1,000 r were necessary to vacuolate nucleoli *in situ*.

(2) 5,000–10,000 r were necessary to initiate removal of lateral chromosome loops. (This was supported by the observation that 4,000 r of *in vivo* radiation of *Triturus* failed to remove lateral loops from chromosomes of large eggs 5 days after treatment.)

(3) At least 10,000 r were necessary to cause occasional fracture of the chromonemata and at least 30,000 r to cause multiple fragmentation.

(4) 50,000 r were necessary to change central nuclear gel into a sol.

The next series of experiments was designed to analyze X-ray effects on separate cell parts. Ideally nuclei, chromosomes, and nucleoli should be irradiated separately and recombined. Experimentally it was possible only to recombine nuclei with cytoplasmic brei. However unsatisfactory as this type of experiment may be, it constitutes an advance over other comparable techniques of nuclear and cytoplasmic isolation.

Tables 4*B* and 4*C* summarize experimental permutations using ground-up irradiated cytoplasmic brei with nonirradiated nuclei and converse exposures of isolated nuclei without the presence of cytoplasm. The results of 8 experiments, run on 3–5 eggs each, agreed in showing that under the conditions of the experiment 50,000 r irradiated brei will cause heavy nucleolar injury, transform spindle substance to a sol, remove and dissolve many lateral loops, but will not cause fragmentation of the longitudinal chromonemata. It should be pointed out that untreated brei has only slightly injurious effects on the nuclear colloid but differs sharply from the irradiated in its effects on chromosomes and nucleoli.

Table 4*C* brings out a result of great significance not generally recognized. This is that nuclei, when isolated, are extraordinarily radioinsensitive. Dosages as high as 30,000 r produced no visible injury, and

double this amount affected only the nucleoli and colloidal suspension system to a relatively limited degree.

Photomicrographs of nuclear conditions are shown in Figure 7, *C* and *D*. It will be noted (Figs. 6, 7, and 8) in the bullfrog egg, that 50,000 r, applied to the whole cell, fragmented the chromosomes in less than 15 minutes. In contrast, the same dosage on the isolated nucleus caused only slight vacuolization and swelling of nucleoli and no obvious damage to chromosomes. In *Rana clamitans*, the green frog, similar radioinsensitivity of an isolated nucleus is shown (Fig. 6, *C*), and in *Triturus pyrrhogaster*, even 70,000 r dissolved only a few lateral loops and produced no chromonema fragmentation when observed only 15 minutes later. Doses of 100,000 r applied to isolated chromosome pairs of salamanders failed to fragment them.

The converse of this condition, photographed in Figure 8, *A* and *C*, illustrates the profound damage resulting when isolated nuclei were replaced in cytoplasmic brei and both irradiated together. It is clear, therefore, that the isolation procedure did not, *per se*, reduce radiosensitivity. All these experiments furnish a sound basis for concluding that nuclear damage results mainly from chemical changes in the cytoplasm. Figure 8, *B*, shows typical changes resulting from placing a nonirradiated *Rana catesbiana* nucleus into the irradiated brei. The shriveled chromosomes pair, and the vacuolated nucleoli are clearly evident. Attention is drawn to the serious derangement of the chromosomes in all three photographs, as contrasted with the normal positions illustrated in the large cell in Figure 7, *A*. Figures 1, 2, and 3 also show the severe damage of radiation to the nucleus *in situ*, as contrasted to nuclear insensitivity when isolated from the cytoplasm. Fragmentation of chromosomes of the bullfrog with 50,000 r is clearly shown at somewhat higher magnification in Figure 7, *C* and *D*.

It is significant that chromosome fragments usually include cross-over loci. At one point designated by the arrow in Figure 7, *D*, the separation of chromatids is evident. In strong support of the above results based on work with frogs, 34 experimental runs on *Triturus pyrrhogaster* threw light on the mechanisms involved. After 3 series, using sets of eggs irradiated at 10-, 20-, 30-, 40-, 50-, 60-, and 70,000 r, which gave the overall changes to be expected, attention was concentrated on the 50,000 r and the 60,000 r doses and their effects on isolated cell components. In general, the types of damage and the magnitude of injuries corresponded closely to anuran data for each dosage level (*cf.* table 4A).

However, in one respect, there was a significant difference between the two types of animals. In the urodeles, nucleolar reactivity was greater in degree, although similar in kind to that of frogs. The coacervate characteristics of nucleoli pointed out in a previous paper (*18*) are of special importance in understanding their behavior. Only the generalizations can be reported here as the details are too numerous and complex. The photomicrographs in Figures 9, 10, 11, and 12 illustrate the

unusually clear type of observations possible in freshly isolated nuclei. It should be kept in mind that equatorial and obverse views are also possible only with this particular technique. A normal set of *Triturus* nucleoli is illustrated in Figure 9. At this stage of the egg's development, they are seen adherent to the inside of the nuclear membrane. It should be noted that they are small globules (9μ–10μ), fairly rounded and regular in shape. Internally there are small vacuoles. Portions of lateral-loop (fuzzy) chromosomes are visible as indicated by the arrow. Figure 10 shows at similar magnification characteristic coacervate changes produced in 2 minutes by treating the whole nucleus with K_2HPO_4 buffered Ringer solution (pH 8.0). Internal vacuoles swelled and burst toward the outside, resembling yeast budding. The resultant picture is usually that of nucleoli in groups of 2 or 3, approximately 18μ–20μ in diameter. The doubled condition of nucleoli, therefore, explains a condition which puzzled earlier cytologists who gave the term amphinucleoli. Attention is further drawn to an observation reported in 1941 (*18*) that chromosomes can rapidly lose lateral loops in mildly alkaline solutions, although the chromonemata seldom break. Disintegrating loops are also to be seen in the photograph. It is of interest that some fragmentation does occur, however, when the phosphate Ringer is rapidly replaced with bicarbonate Ringer at pH 7.6.

Figures 11 and 12 show the effects of irradiation on nucleoli at comparable magnification as Figures 9 and 10. The photomicrograph in Figure 11 is a top view of the nucleus irradiated after isolation, while that in Figure 12 is a similar view of the nucleus irradiated inside the cell and isolated immediately afterwards. Both received 50,000 r. It was surprising that the nucleoli from the former (Fig. 11) showed such small deviations from the normal (Fig. 9), while those irradiated in the cytoplasm (Fig. 12) showed such striking changes. From comparison of photomicrographs in Figures 10 with 12, it must be concluded that similar colloidal changes in the nucleoli can be produced both by radiological and simple chemical means.

From analysis of the experiments on *Triturus pyrrhogaster* eggs, four more conclusions may be added to those presented above:

(1) Urodele egg cells respond to radiation in essentially the same ways as anuran eggs. Therefore, effects are not species specific (*cf.* table 4A).

(2) Isolated nuclei are markedly radioresistant up to the 30,000 r level, and only slightly responsive up to the 60,000 r level.

(3) Irradiated cytoplasmic brei (50,000 r) has the power to vacuolate nucleoli, transform spindle substance from gel to sol, and cause lateral loop disintegration.

(4) Most of the criteria of nuclear radiation injury can be duplicated by exposure to simple chemical solutions.

Thus, the evidence is strongly in favor of the hypothesis that radiation injury to nuclei is not direct, but indirect, and mediated by chemical

changes in the cytoplasm. For more conclusive proof of this hypothesis, attention is directed to the next section.

3. Nuclear Injury From Injection of Irradiated Cytoplasm

Previous sections of this paper have served to establish criteria for nuclear damage *in vivo* and to indicate the indirect mechanism involved by use of cellular components treated separately and in recombination. A further direct experimental approach was needed to prove that cytoplasm from an irradiated cell contains a substance or substances injurious to normal nuclei. Within the limits of the criteria established, the microinjection technique described earlier answered this need.

It was first necessary to find what limits of manipulation the eggs would sustain and what were the nuclear events following injection of cytoplasm from ovarian eggs of one animal into the cytoplasm of another under physiological conditions. From preliminary trials it was found that cytoplasmic material could, in spite of certain technical difficulties, be withdrawn from the transparent eggs of frogs, salamanders, and fish. So-called hyaloplasm with suspended granules of less than 1μ and pigment granule aggregations would swirl into the pipette opening under reduced pressure. The nucleus always moved slowly toward the point of the withdrawal; on occasion a strand of nuclear membrane would be sucked into the pipette tip, plugging it, and cancelling the experiment. The shrinkage of cell volume was routinely measured with an ocular micrometer. Amounts of cytoplasmic material withdrawn were of the order of 0.0001 mm.^{3} Injection of host cells was considerably more difficult, because each pipette tip was already broken. Of 98 experiments, 35 percent were failures from this cause. However, in the other 65 percent, injections were physiologically successful. Cytoplasm could be observed mixing with that of the host. Usually the injection mass flattened the host nucleus temporarily, as the cell swelled, and often the nucleus would rotate. Occasionally some of the host cytoplasm leaked around the injection tip, but usually the hole sealed over quickly, and the cell resumed its normal appearance.

In 30 out of 35 cases of successful injections of normal cells by normal cytoplasm from another animal's egg, no nuclear change resulted, even after 2 hours. In 5 instances, however, nuclei became granular in 12–47 minutes and finally developed coagulated nuclear bodies (C. N. B.). This is interpreted as meaning that nuclear injury involving pyknosis is essentially a basic type of cellular death process and can result from mechanical or other injury. However, the fact that 86 percent of the injections with normal cytoplasm produced no visible nuclear damage whatsoever, and the fact that injections of frog cytoplasm into salamander eggs and vice versa also produced no observable injury, can be taken as a baseline for the following experiments. A photomicrograph of the process of injection and the appearance of normal cytoplasm after transfer is shown in Figure 13. Forty-one experiments were attempted using irradiated

ovocytes as donors and eggs of control animals as hosts, but only 29 were physiologically successful. *Triturus viridescens* was used throughout this series. Irradiations of 3,000 r were given, since this dosage had previously been found effective when followed by a period of 5–7 days at room temperature to allow injuries to develop. Donor cells were selected with pronounced central nuclear bodies and granular cytoplasm. It also served to identify the host cells. Eleven experiments were unsuccessful either because of pipette failure or because the subsequent behavior of the cell could not be followed for sufficient periods. On the other hand, 23 of 29 successful injections produced sharply defined central nuclear bodies and other injury effects, similar in every detail to pyknotic changes seen in the direct radiation experiments. Two others produced central granular areas. It is regarded as significant that these changes did not begin immediately but developed gradually after 10 minutes to half an hour.

The experimental production of pyknosis was studied in detail. First evidence of nuclear injury was slow swelling of the nucleoli. During the early period, the centers of the nuclei remained entirely clear, with chromosomes hydrated and, therefore, invisible as in normal living cells. After 10–30 minutes, slight granulation began to appear centrally. As this developed, it became apparent that the expanded chromosome frame was contracting, drawing the chromosomes centripetally. At the same time nucleoli often everted their contents through the nuclear membrane into the cytoplasm, forming multiple pinkish spherical vacuoles on the entire outer surface of the nucleus. The final stage of pyknosis began with the formation of an irregular central mass that sank to the bottom of the nucleus. Here it was seen always covered with large vacuoles, as illustrated in Figure 17. These central nuclear bodies roughly 50 μ in diameter, were identical with those illustrated in Figure 4, produced *in vivo* by X-irradiation. They were also identical with those produced by β-radiation from radioactive phosphorus as will be described in another paper. Examples of various stages of nuclear injury as followed in the same cell are shown in Figures 14, 15, 16, and 17. In each nucleus with a precipitated central nuclear mass there were observed many fragmenting granules (1μ–2μ) in the space formally occupied by the chromosome frame. Linear connection could not be demonstrated between these disintegrating particles, although limited Brownian motion suggested they might still be attached to elastic and microscopical threads.

Cytoplasmic changes following injection were more difficult to observe. In four cases only definite coagulation of the injected mass proceeded slowly. Examples are shown in Figures 15 and 16. However, in the large majority of cases, injected cytoplasmic masses mixed with the host cytoplasm so completely that identity of each was lost. Since flocculation of the cytoplasm routinely occurred in heavily irradiated eggs, the parallelism has been noted. Cytoplasmic injections in successful experiments showed no evidence of abnormal Brownian motion. A significant fact

emerging from study of 3,000 r irradiated animals maintained for unusually long periods in the cold (21–26 days) is that all the eggs showed extensive cytoplasmic damage with nuclear damage only beginning. An example is given in the photomicrograph of Figure 18. Cytoplasm injected from these cells produced typical pyknosis in nonirradiated hosts, although their own nuclei were relatively undamaged. Thus, a physiological test has been established for the presence of the nuclear toxin.

From the above experiments the overall conclusion is inescapable that irradiated cytoplasm when injected into normal cells can induce typical nuclear injury. It is important to bear in mind that this injury is of a nonspecific but general type which can occur under other conditions.

DISCUSSION

No theory of radiation damage is adequate unless it explains or attempts to explain differential susceptibility of similar cells exposed to statistically equal ionization densities. As indicated below, the metabolic factor meets this challenge in that it allows for a varying differential based on both quantities and qualities of cytoplasmic toxin produced. The data already presented have included new differential criteria for visible nuclear damage and new approaches to radiation injury mechanisms. It has been shown, for example, that chromonemata are more resistant than lateral loops, that nucleoli react differently when radiated *in situ* as compared with behavior after isolation, that colloidal chromosome suspension system can be changed, that low temperatures retard nuclear injuries, and that irradiated cytoplasm injected into normal cells can produce pyknosis. It has been demonstrated that cytoplasmic intervention plays an important role in all of these reactions. Attention is drawn to the observations that isolated nuclei are relatively insensitive to X-rays, whereas either by exposure to an irradiated brei, or by injection of irradiated cytoplasm, nonirradiated nuclei can be made to react.

The great dependence of visible nuclear injury upon temperature proves that at least one link in the chain of events has a high temperature coefficient. On the other hand, data from Patt and Swift conclusively show that the temperature at which radiation is given frogs is immaterial compared with the temperatures at which they are subsequently maintained. From a physical viewpoint, it is also clear that primary ionization is independent of temperature. Although the experiments of Evans, Goodrich, and Slaughter (*20*) seem to stress the importance of temperature at the time of radiation, it nevertheless appears to be a sound assumption in these experiments that the radiochemical, instantaneous events were independent of temperature. The observed temperature influences were imposed later and, therefore, must have been acting on secondary metabolic processes. It should be pointed out that it would be difficult, if not impossible, to irradiate cells in the cold and the next instant have them metabolizing normally at, say 22° C. Consequently, we must remain skeptical of results of others who have claimed to separate these factors.

It is best to conclude, therefore, that the primary (physical) effect of irradiation is not sensitive to temperature.

Facts have been brought forward to show that while most nuclear damage is delayed by the cold, nevertheless some appeared while the animals were kept for long periods on ice. This is interpreted to mean that metabolic factors were in control. Conversely, the few animals maintained at warmer temperatures (26°–27° C.) showed acceleration of nuclear injuries. It is significant that all the egg cells in animals exposed to 3,000 r showed cytoplasmic damage after 3 weeks at low temperatures and that nuclear damage did not appear in quantity until after warming. Nuclear injury of germ cells was practically absent in an individual shielded locally with lead. It is concluded, therefore, that nuclear irradiation injury is metabolically governed by forces acting within the ovarian region. It is most probable that these forces are damaged enzyme systems in the cells themselves [*cf.* Barron (*10*)].

Finally it has been shown that nuclear injury results both *in vivo* and *in vitro* from close contact with irradiated cytoplasmic material. The reactivity of nucleoli, chromosomes, and colloidal suspension material to the environment was established. Exposure periods, in an altered cytoplasmic environment, of 10 minutes to an hour are necessary to produce nuclear changes. It is inescapable that some sort of diffusion process must be involved. Such transmission processes would be expected to be affected by localized protoplasmic viscosity differences to a much greater extent than by temperature. In over 95 percent of the cells irradiated with 5,000 r or higher, and in 90 percent of those given 3,000 r and kept alive 4 days, there was localized cytoplasmic clumping as well as areas of liquefaction.

The data presented in this paper are consistent in showing that different components of the nucleus have different thresholds to X-irradiation: at least 5,000 r were necessary to vacuolate the nucleoli, 10,000 r to fracture the longitudinal chromosome threads (chromonemata), and 50,000 r to change the central gel into a sol. Comparable effects were obtained with microinjections of cytoplasm which had received only 3,000 r. It was further demonstrated that primary cellular injury varies with increasing dosages. In each instance a cell exposed to heavy irradiation became involved in a qualitative and quantitative chain of events which may fairly be termed toxicological.

Other than experiments with variants of simple ion systems, no attempt has been made to identify the toxic chemicals responsible for nuclear damage. They could be normal katabolic products in abnormal quantities. An extensive literature on this subject already exists (*1*, *9*). For example, Hevesy (*5*) has examined diminished turnover of phosphatides after X-radiation. Knowlton and Hemplemann (*21*) have endeavored to quantitate sublethal doses of irradiation on mitotic activity of specialized cells but were unable to arrive at a theoretical explanation of the results. On the other hand, Zirkle (*2*) has recently made a theoretical analysis of

relationships between chemical and biological effects of ionizing radiations. In reference to the need for relating a specific radiochemical reaction to a biological effect, he states, "nobody has yet demonstrated flocculation of proteins in an (irradiated) cell." A correlation of meiotic stages in ovarian eggs of *Habrobracon* to X-ray sensitivity has been developed by Whiting (*22*). Her observations support the conclusions here, *i. e.*, that older, larger amphibian eggs are less sensitive to radiation injury than the small transparent ones. It is known that mice irradiated with an acute sterilization dose can produce one additional litter of young but none thereafter. It is suggested that one reason why the larger nuclei of older and bigger eggs do not respond as fast to irradiation is because of the greater nuclear and cytoplasmic volume ratios to the quantity of cytoplasmic toxin produced.

As pointed out in the beginning, the need in experimental pathology is for new criteria of damage in a cell rather than new theories of damage. It is with some reluctance that another hypothesis is offered in this paper. Cytochemical events are so complex that all theories regarding any of them should be regarded as tentative and subject to alteration. The comment may be offered that any experiment purporting to differentiate between radiation effects in the nucleus from those in the cytoplasm should involve actual physical separations, as done here. Experiments on centrifuged eggs or irradiation of sperms *vs.* eggs do not rigorously fulfill these conditions, and so must remain equivocal. The direct approach is surely preferable.

The data presented here support the general conclusions of Patt and Swift (*11*) that "primary radiation is not influenced by the metabolic level and that it is not repairable in the metabolically depressed animal." They have further demonstrated that primary cellular injury varies with increasing dosage. It has been demonstrated that the cause of visible nuclear injury originates primarily in the cytoplasm and not in the nucleus, although most radiologists agree that nuclear changes are the best evidence of damage. Thirdly, it has been found that the toxic principle is transmitted into the nucleus and that the nuclear membrane is involved. In summary, the following theory of radiation injury is offered. It consists of three parts: (1) Production of localized radiochemical events (involving damage to sensitive molecules, such as enzymes, and possible formations of peroxides). These are apparently temperature insensitive since they are instantaneous occurrences at the molecular level. (2) Accumulation of toxic katabolic substances rather more than production of new toxic substances of intermediate metabolism. Because these involve chain reactions that might be interrupted or delayed unevenly, products at successive substrate levels would be variable both as to time and quantity. It would be expected that these secondary events are highly temperature sensitive. (3) Transmission of toxic products or accumulations in various parts of the cell including the nucleus. Observable nuclear reactions have been found to depend on

diffusion and should be more affected by local viscosity changes than by temperature.

This cytochemical hypothesis is not original or new. But in its present form it has some advantages over other theories, such as those reviewed recently by Dobson and Lawrence (*23*). It furnishes explanations for the lag period in radiation injuries and the problem of differential susceptibility in cells. It can account for the generalized effects on nucleoli and chromosomes, rather better than the target theory, with which it does not conflict. And it can explain the earlier and greater injury effects in smaller eggs and frequent lack of effects in larger ones. Finally, it offers another experimental approach to the problem of deeper nucleocytoplasmic relationships on which a study has been begun with the aid of radioactive tracers (*24*).

SUMMARY

Ovarian eggs of salamanders and frogs, when irradiated with X-ray doses from 500 r to 60,000 r in graded steps, showed progressive stages of pyknosis. With 3,000 r, 98 percent of the small eggs showed intranuclear damage within 10 days.

New criteria of radiation injury in amphibian eggs included: fragmentation of the chromosomes, loss of lateral chromomere loops, colloidal changes in the nucleoli, sol-gel changes in the intranuclear spindle substance, and localized cytoplasmic flocculation.

Nuclear components irradiated *in situ* showed differential susceptibility. Nucleoli were vacuolated at 1,000 r, chromomere loops were dissolved after 2,000 r, chromonemata were broken by 3,000 r, and spindle substance was solvated by 50,000 r. However, when these components were isolated from their cytoplasmic environment and then irradiated, all were markedly radioresistant.

Exposures to low temperature (5° C.) delayed nuclear injury up to 18 days postirradiation but were unable to prevent pyknosis from appearing when animals were rewarmed to 23° C. Higher temperature (27° C.) accelerated the rate of damage production.

Nuclear and chromosomal injuries in ovarian eggs were roughly paralleled by those in theca cell (somatic) nuclei. Effects were localized in the cells studied and were not systematic in origin.

Typical nuclear radiation damage in nonirradiated cells could be produced by microinjections of cytoplasmic material withdrawn from irradiated eggs. Chromosomal and nucleolar injuries could likewise be produced by exposure of isolated normal nuclei to irradiated cytoplasmic brei.

The conclusion is drawn that cellular radiation damage is a triple phenomenon consisting of: primary physical or radiochemical changes in the cytoplasm; chemical metabolic processes which allow protoplasmic nuclear toxins to form or accumulate in the cytoplasm; and transmission of toxic substances into the nucleus in 10–30 minutes at 23° C.

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Plates

PLATE 108

FIGURE 1.—Portion of ovarian fragment showing typical nuclear X-ray damage in three small transparent eggs. Two larger eggs still have normal nuclei. *Triturus viridescens*, 3,000 r, 2 days postirradiation. × 200.

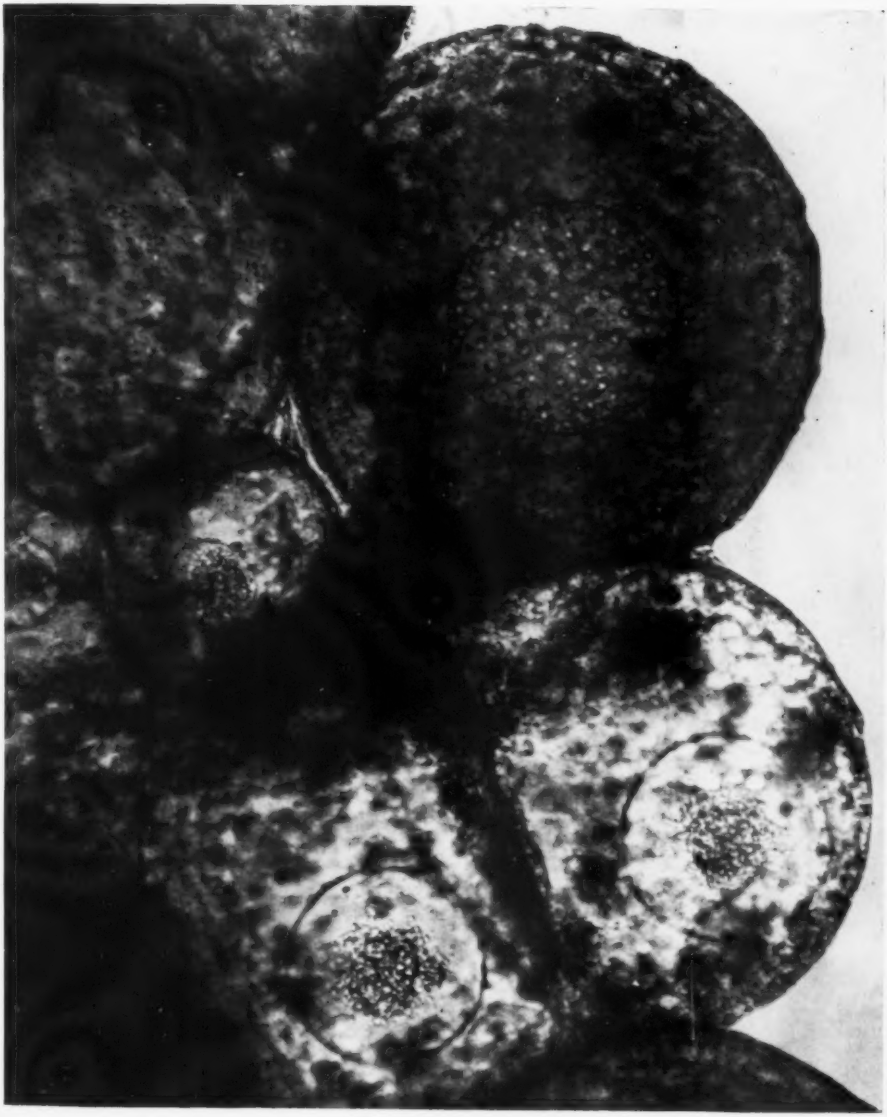


PLATE 109

FIGURE 2.—Irradiated transparent egg, showing clumped and stretched chromosomes, partial loss of lateral loops, nucleoli everting through nuclear membrane. Several theca cell nuclei with clumped chromosomes are also in focus. *Triturus viridescens*, 3,000 r, cold 7 days, warm 2 days. × 880.

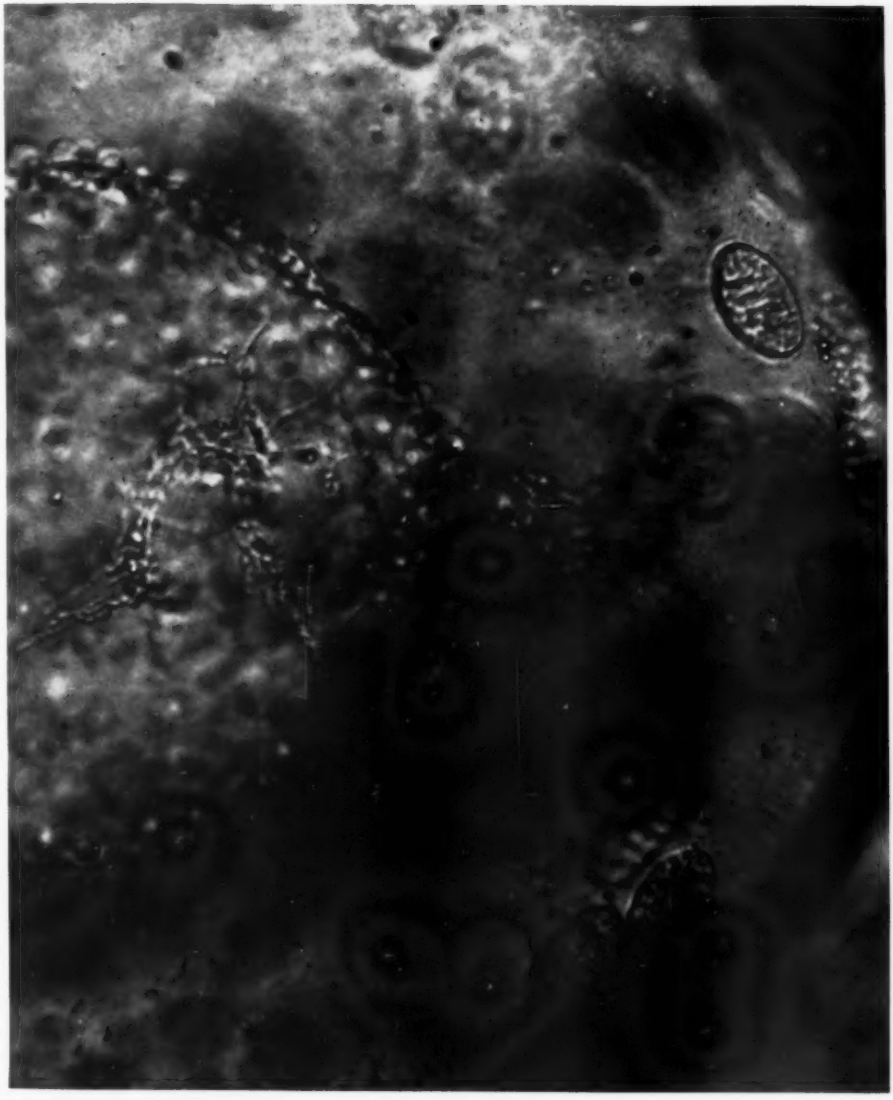


PLATE 110

FIGURE 3.—Details of irradiated chromosomes in nucleus of transparent cell. Note central clumping, disintegration of lateral loops, and stretched chromonemata. Chromosomes appear to be coated with viscous fluid. Nuclear colloids are undergoing phase separation. *Triturus viridescens*, 3,000 r, 2 days postirradiation. × 930.

PLATE 111

FIGURE 4.—*A*, Beginning nuclear irradiation damage. Chromosomes clumping toward center. Slight swelling at some loci. Nucleoli still normal. Note flocculation in cytoplasm. *Triturus viridescens*, 3,000 r, cold 9 days, warm 2 days. × 650. *B*, Advanced nuclear irradiation damage. Portion of ovarian wall with two transparent eggs. Cell at right shows nuclei with swollen chromosomes. Linearity nearly lost. Cell at left shows pyknosis with marked central nuclear body (CNB). *Triturus viridescens*, 3,000 r, 2 days postirradiation. × 615.

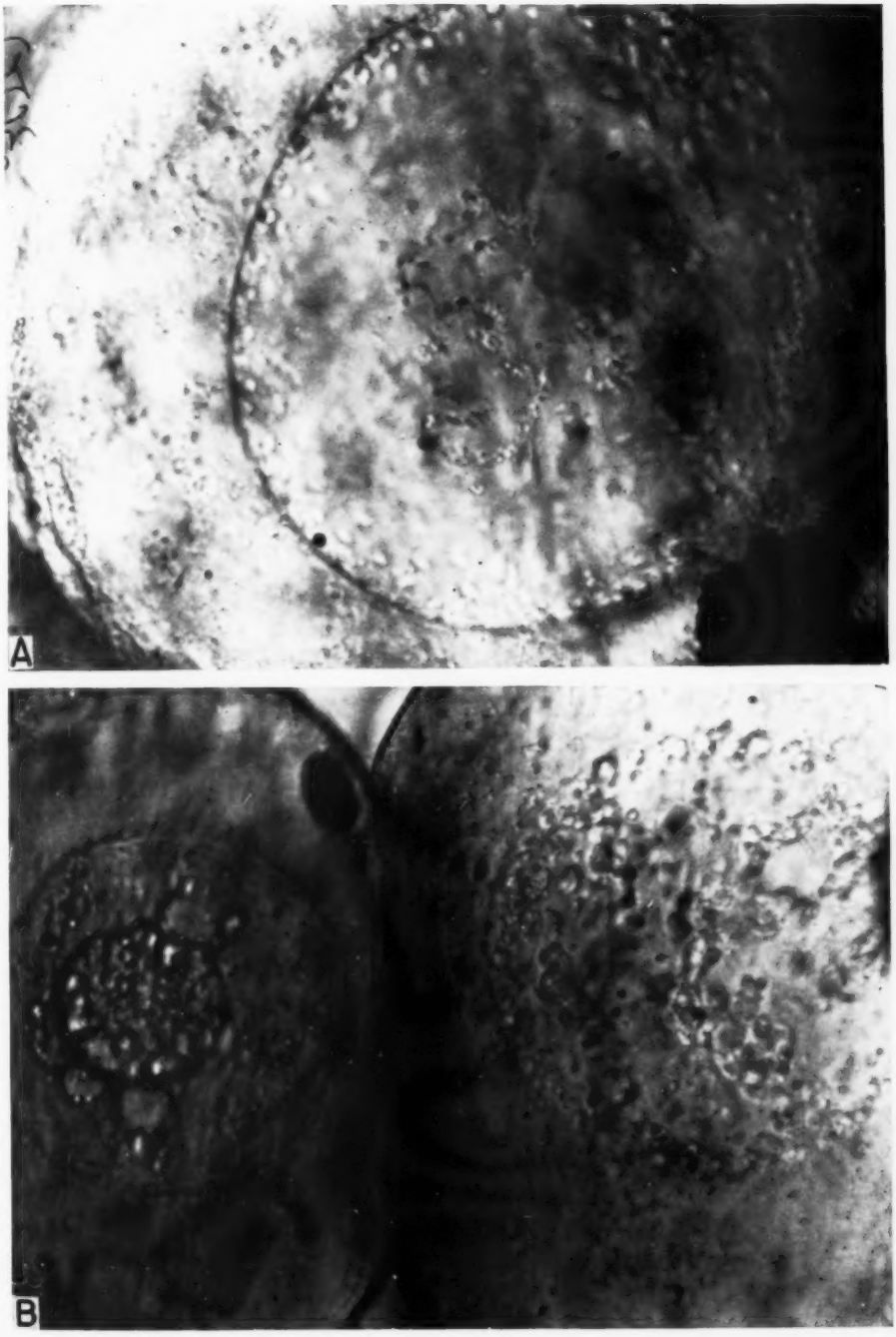
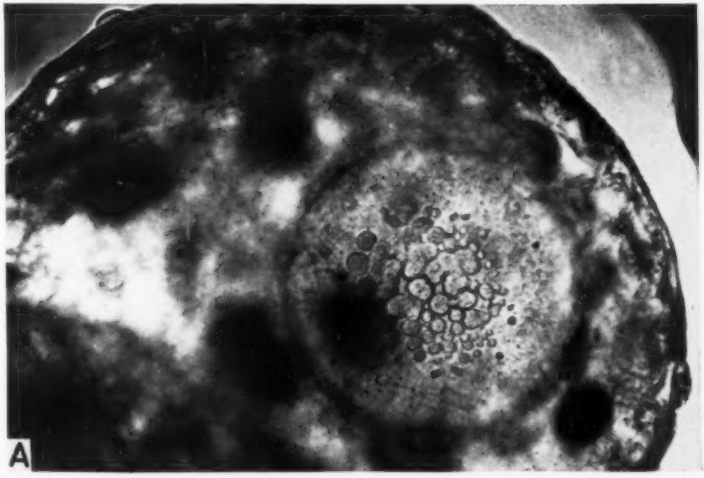
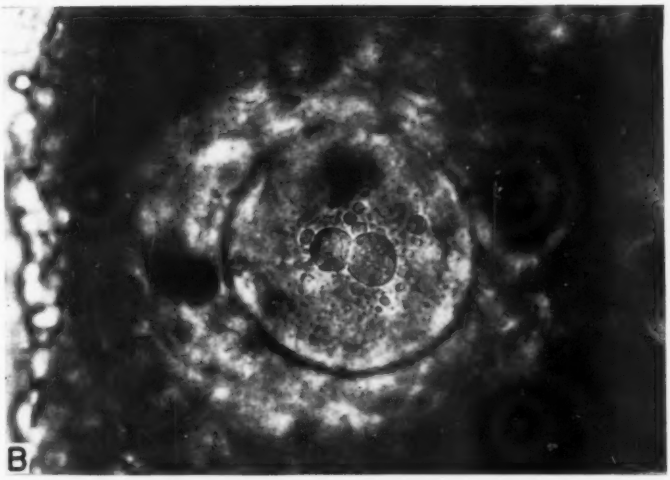

A
B

PLATE 112

FIGURE 5.—*A*, Advanced irradiation damage. Pyknosis proceeding by aggregation of chromosomal fragments. *Triturus pyrrhogaster*, 3,000 r, 96 hours post-irradiation. × 260. *B*, Another egg from same ovary. × 260. *C*, Final aggregation stage of chromosomal droplets to form "central nuclear body." Note internal vacuole and nucleolar remnants. Egg from same ovary as *A* and *B*. Mounted in amphibian Ringer solution. × 260.


A


B

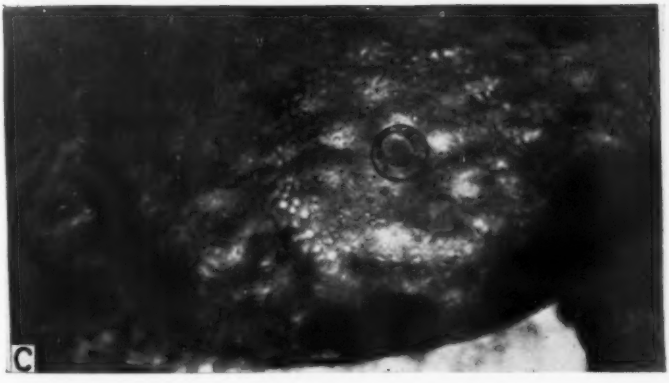
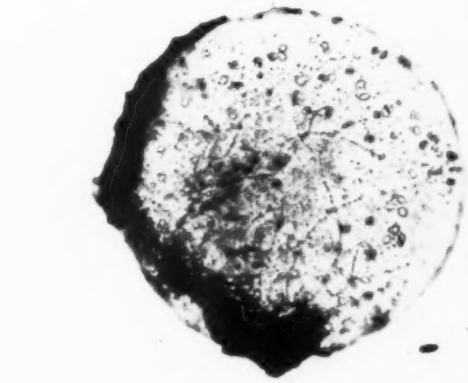
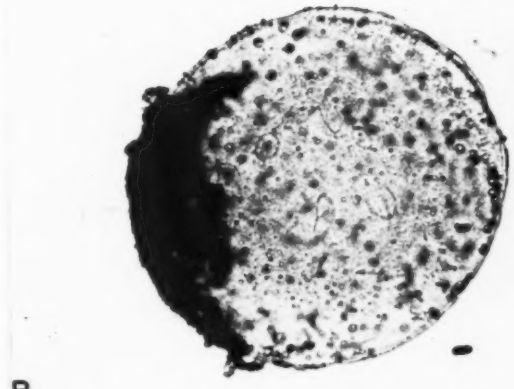

C

PLATE 113

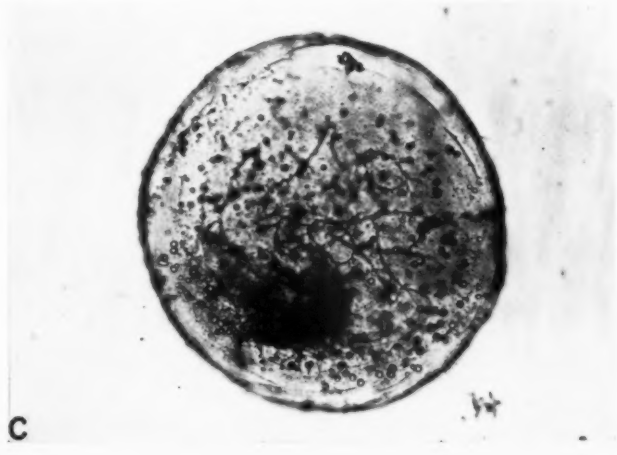
FIGURE 6.—*A*, Normal isolated nucleus in N-medium. Long chromosome pairs throughout nucleus. Dark cytoplasmic remnant attached to outside of nuclear membrane. *Rana catesbiana*. × 120. *B*, Egg nucleus irradiated in cell and then isolated. Note multiple fragmentation of chromosomes. Same nucleus shown in Plate 114, *C*, at higher magnification. *Rana catesbiana*, 50,000 r, 15 minutes postirradiation. × 120. *C*, Egg nucleus irradiated after isolation. Note long chromosome pairs and similar appearance to normal in *A*. *Rana clamitans*, 50,000 r, 20 minutes postirradiation. × 120.



A



B



C

PLATE 114

FIGURE 7.—*A*, Nonirradiated control nucleus isolated in N-medium. Long chromosome pairs have normally shed lateral loops. *Rana catesbiana*. × 230. *B*, Beginning irradiation damage. Nucleus irradiated *in situ*. Slight evidence of fragmentation. *Rana catesbiana*, 2,000 r. × 230. *C*, Heavy irradiation damage. Multiple fragmentation of chromosomes. Nucleus irradiated in situ before isolation. *Rana catesbiana*, 50,000 r. × 230. *D*, Heavy irradiation damage. Multiple fragmentation. Arrow indicates separated chromatids. Note doubled and everted nucleoli. *Rana catesbiana*, 50,000 r. × 230.

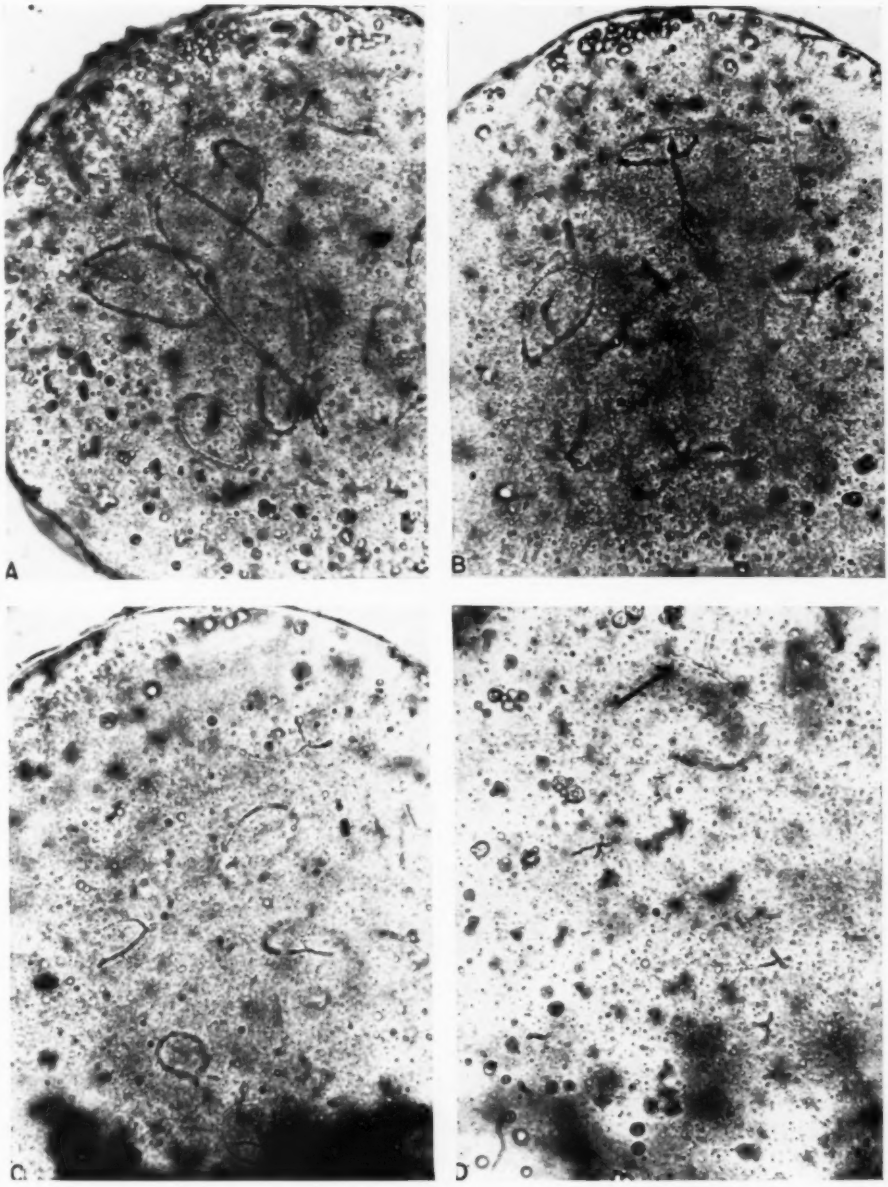
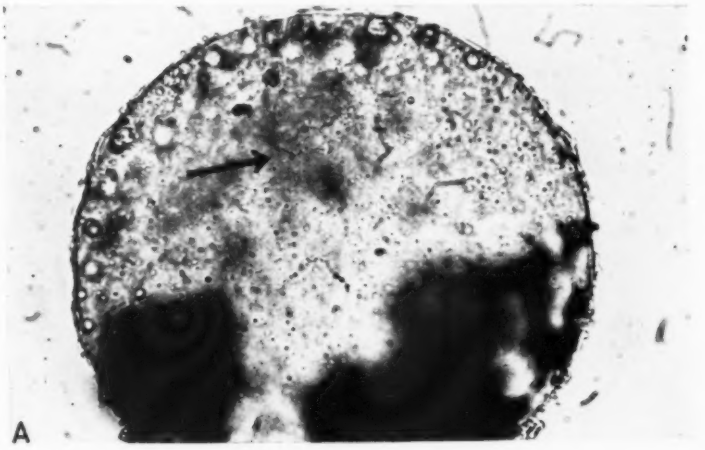
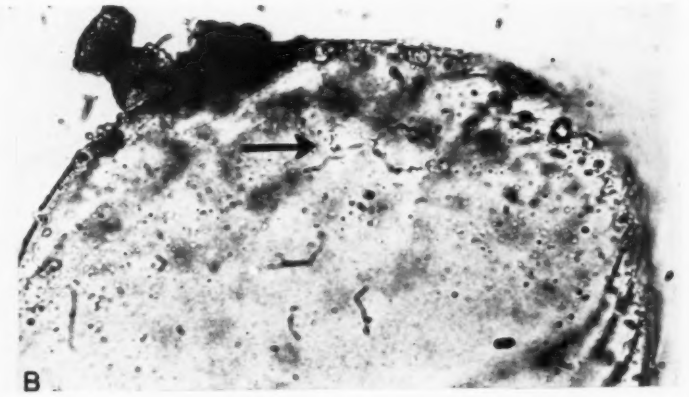

A
B
C
D

PLATE 115

FIGURE 8.—*A*, Effect of radiation after isolation. Nucleus first isolated and mixed with cytoplasmic brei. Both irradiated together. Note clumped, shriveled, and displaced chromosomes indicated by arrow. Compare with Figure 7, *A* and *C*. *Rana catesbiana*, 50,000 r. × 270. *B*, Nonirradiated nucleus exposed to irradiated cytoplasmic brei for 1 hour. Note shriveled and displaced chromosome pair. Lateral loops dissolved. *Rana catesbiana*, 50,000 r. × 270. *C*, Another nucleus irradiated after isolation but with cytoplasmic brei present. Note dark cytoplasmic clump adherent to outside of nuclear membrane. Arrow indicates contracted chromosome pair with lateral loops dissolved. *Rana clamitans*, 50,000 r. × 270.


A


B

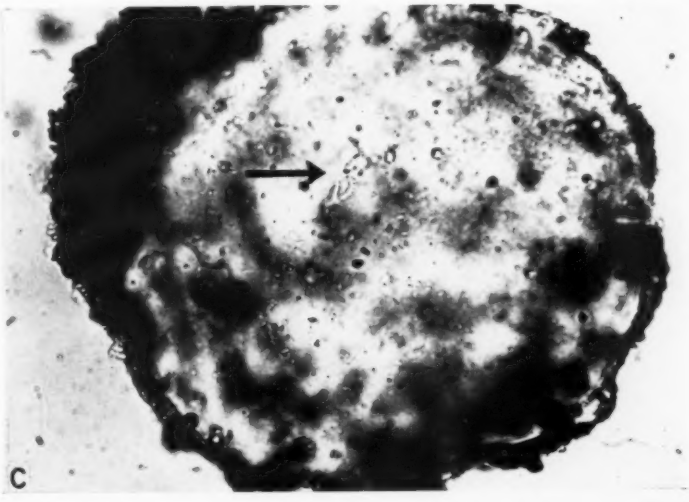

C

PLATE 116

FIGURE 9.—Surface view of a portion of the membrane of a normal isolated nucleus in Ca-free unbuffered Ringer (N-medium). Note regular outlines of most nucleoli. Arrow indicates portion of chromosome with lateral loops. *Triturus pyrrhogaster*. × 820.

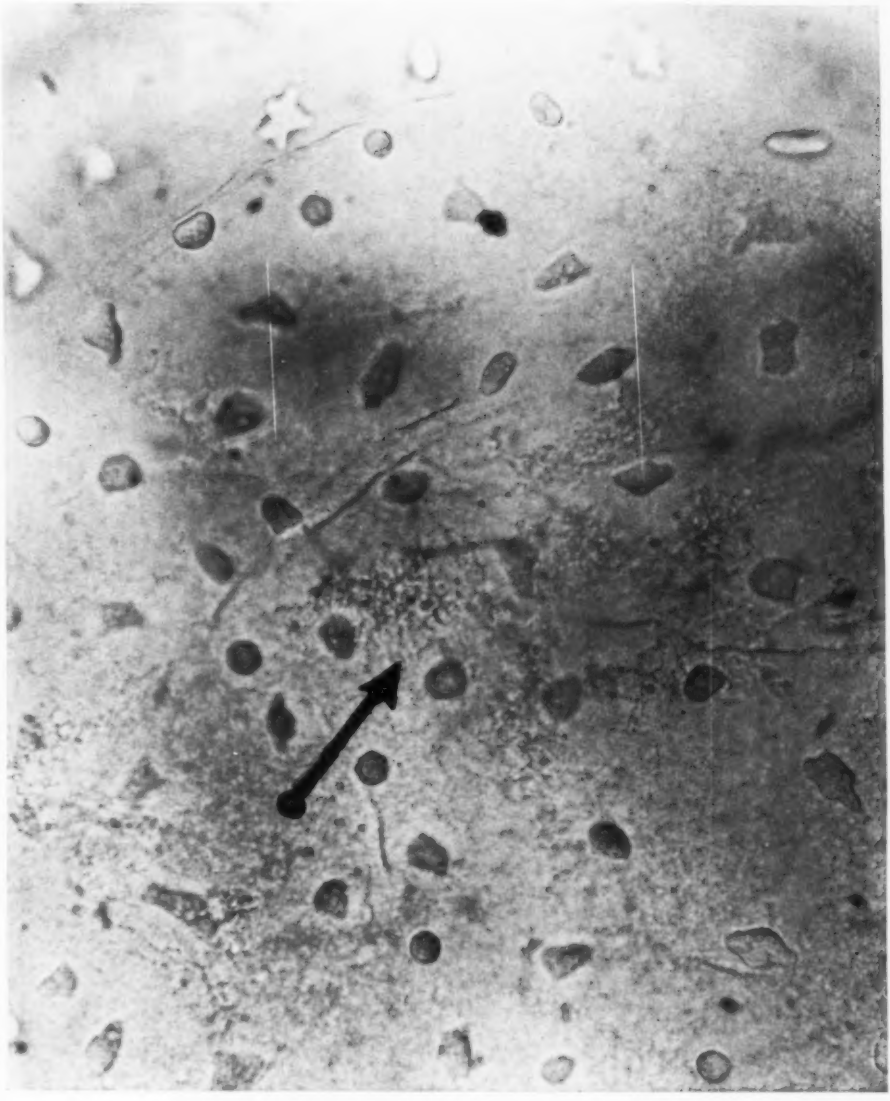


PLATE 117

FIGURE 10.—Surface view of nonirradiated isolated nucleus exposed to alkaline Ringer, pH 8.0. Note enlarged and vacuolated nucleoli. Many have contents everting producing double or triple nucleoli. Stretched chromosome with dissolving lateral loops visible beneath surface. *Triturus pyrrhogaster.* × 890.

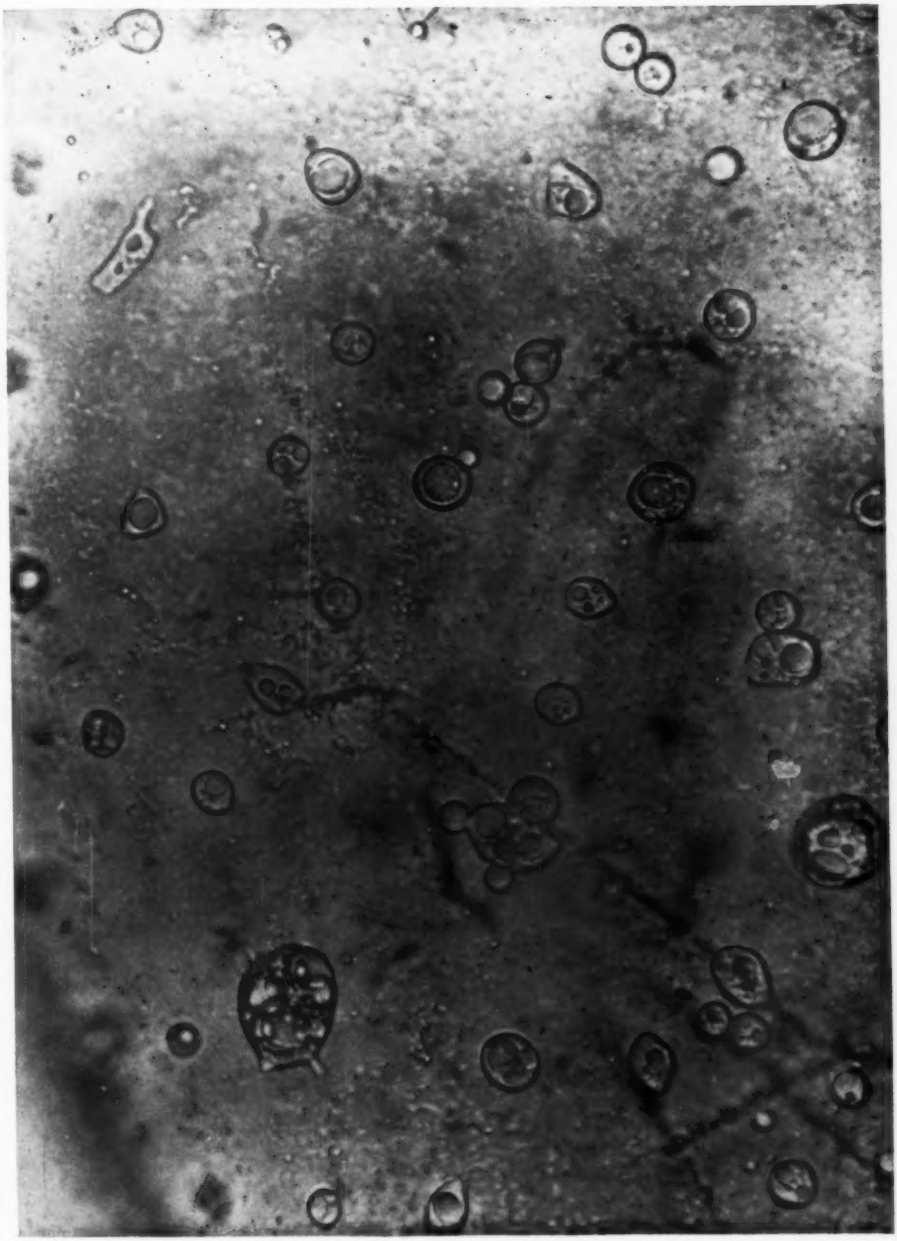


PLATE 118

FIGURE 11.—Radioinsensitivity. Surface view of a nucleus irradiated after isolation. Note general similarity to control in Figure 9 and contrast with Figure 10. *Triturus pyrrhogaster*, 50,000 r. × 890.

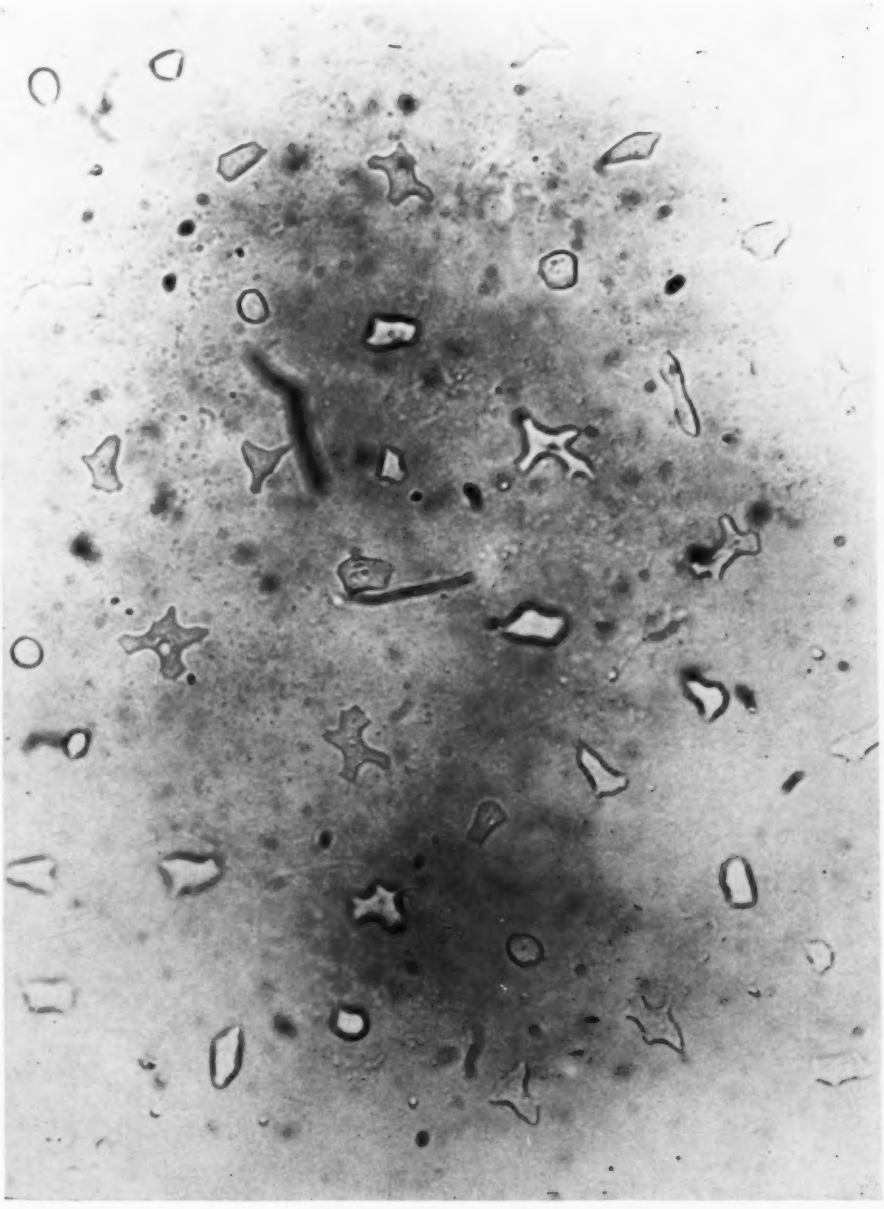


PLATE 119

FIGURE 12.—Radiosensitivity of nuclear component. Surface view of isolated nucleus irradiated in the cell before removal. Nucleoli are greatly enlarged and vacuolated. Compare with Figures 9, 10, and 11. *Triturus pyrrhogaster*, 50,000 r. × 900.

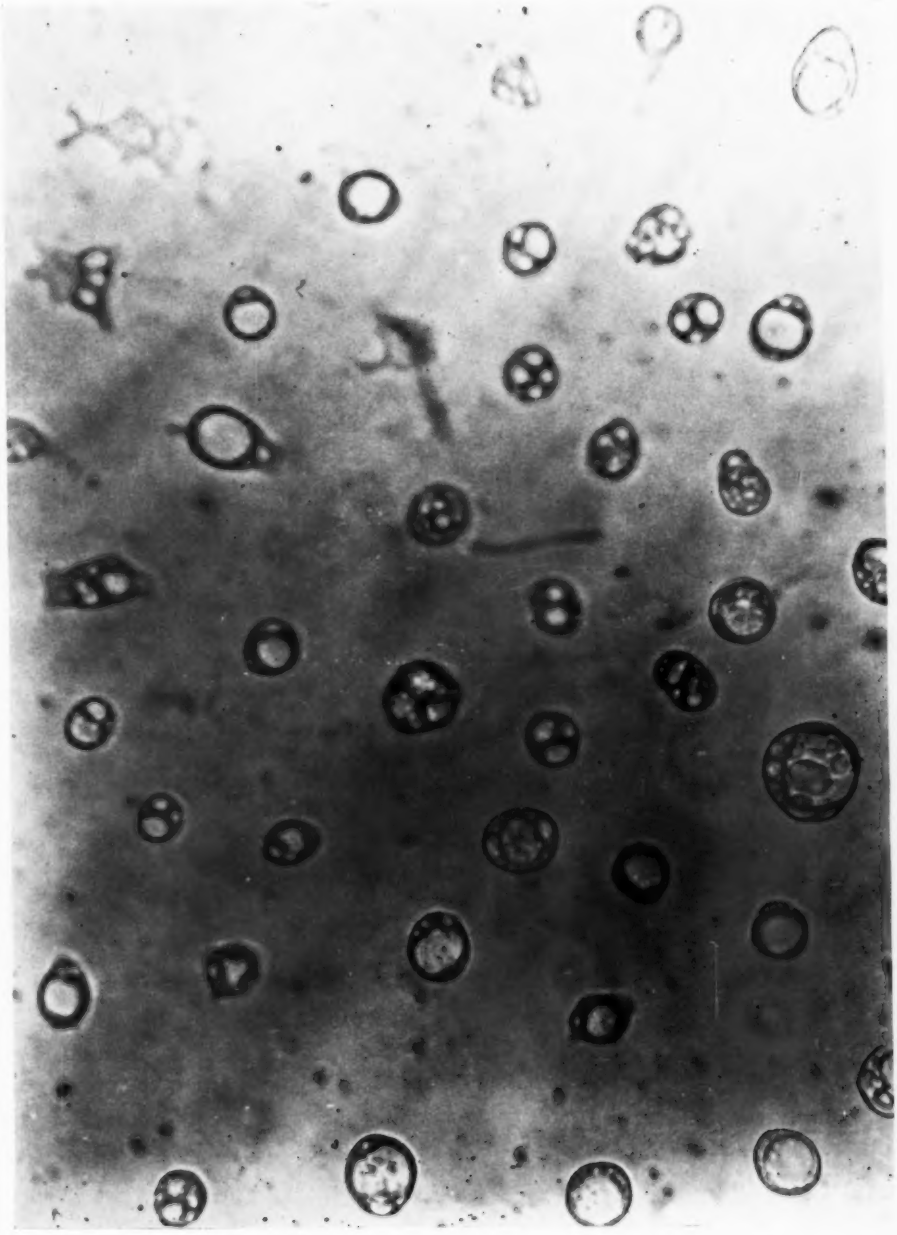


PLATE 120

FIGURE 13.—Microinjection of normal cytoplasm into transparent eggs. Cell at lower right with normal nucleus (control). At upper right micropipette tip visible in cell (arrow). Injection mass flattening nuclear membrane. At upper left cell being held in place with microneedle has already received granular crescentic injection mass of cytoplasm previously withdrawn from egg of another animal. Dark patches are normal pigment masses. *Triturus viridescens*. × 350.

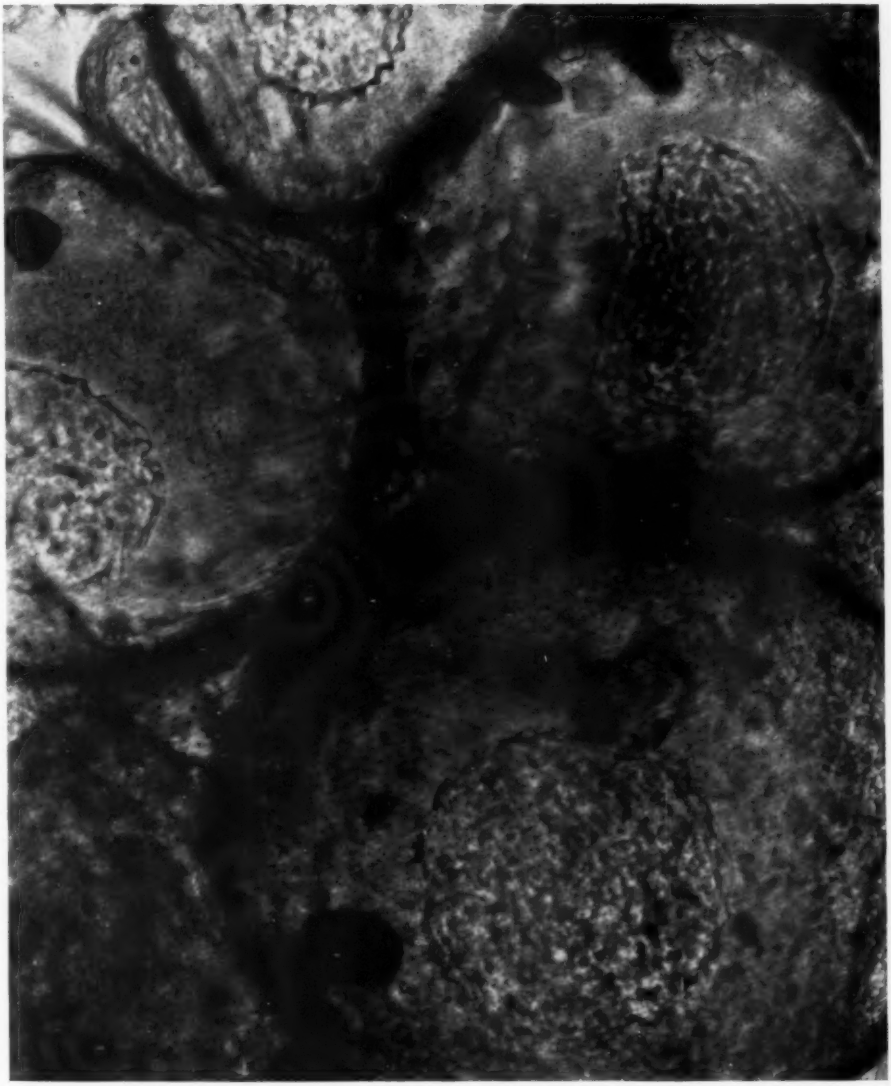


PLATE 121

FIGURE 14.—Microinjection of X-rayed cytoplasm into normal cell. Three minutes post injection. Host nucleus still normal. Note granular cytoplasm from egg of animal irradiated with 3,000 r 7 days previously is surrounding nucleus. Compare with Figures 13 and 15. *Triturus viridescens*. × 800.

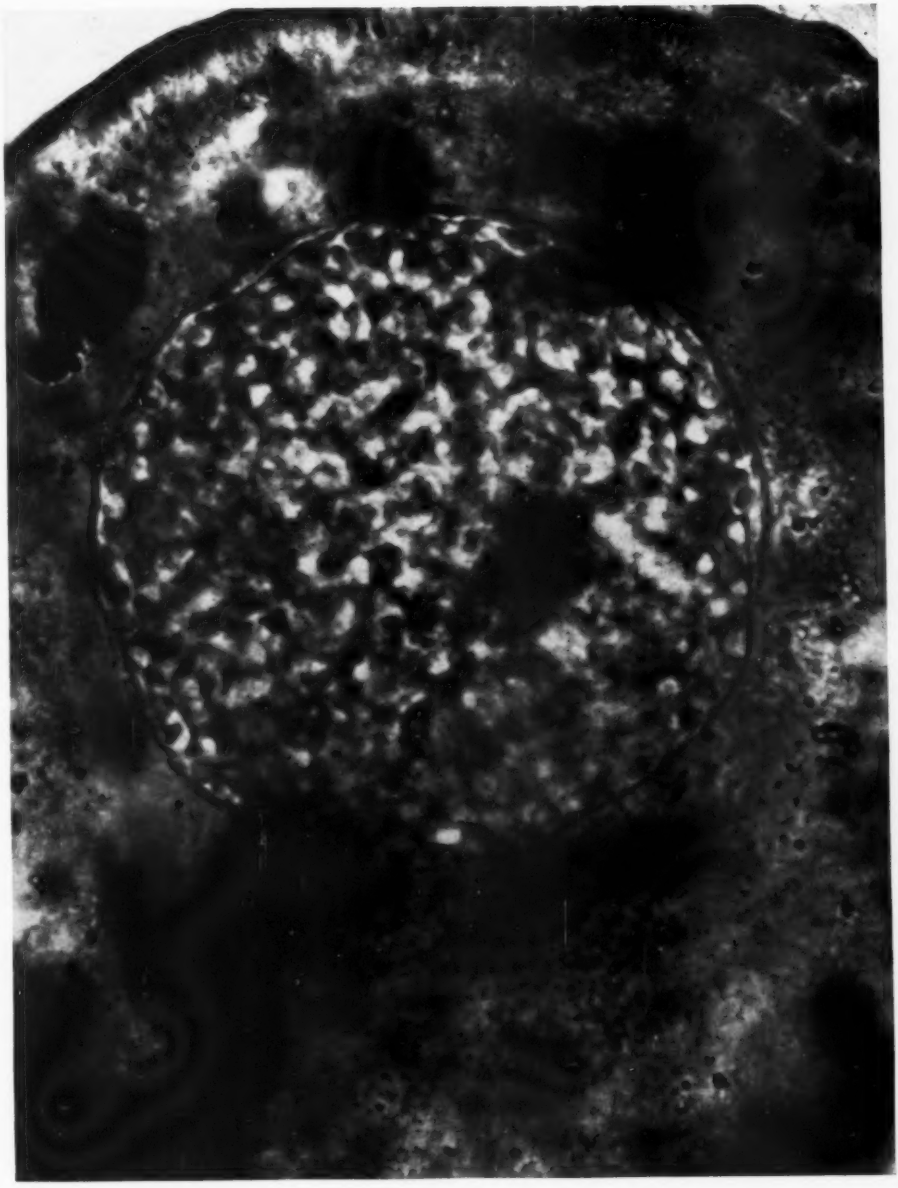


PLATE 122

FIGURE 15.—Same cell as in Plate 121, 38 minutes post injection. Note central spherical mass forming in nucleus and flocculating cytoplasm on surface of nuclear membrane. *Triturus viridescens.* × 800.

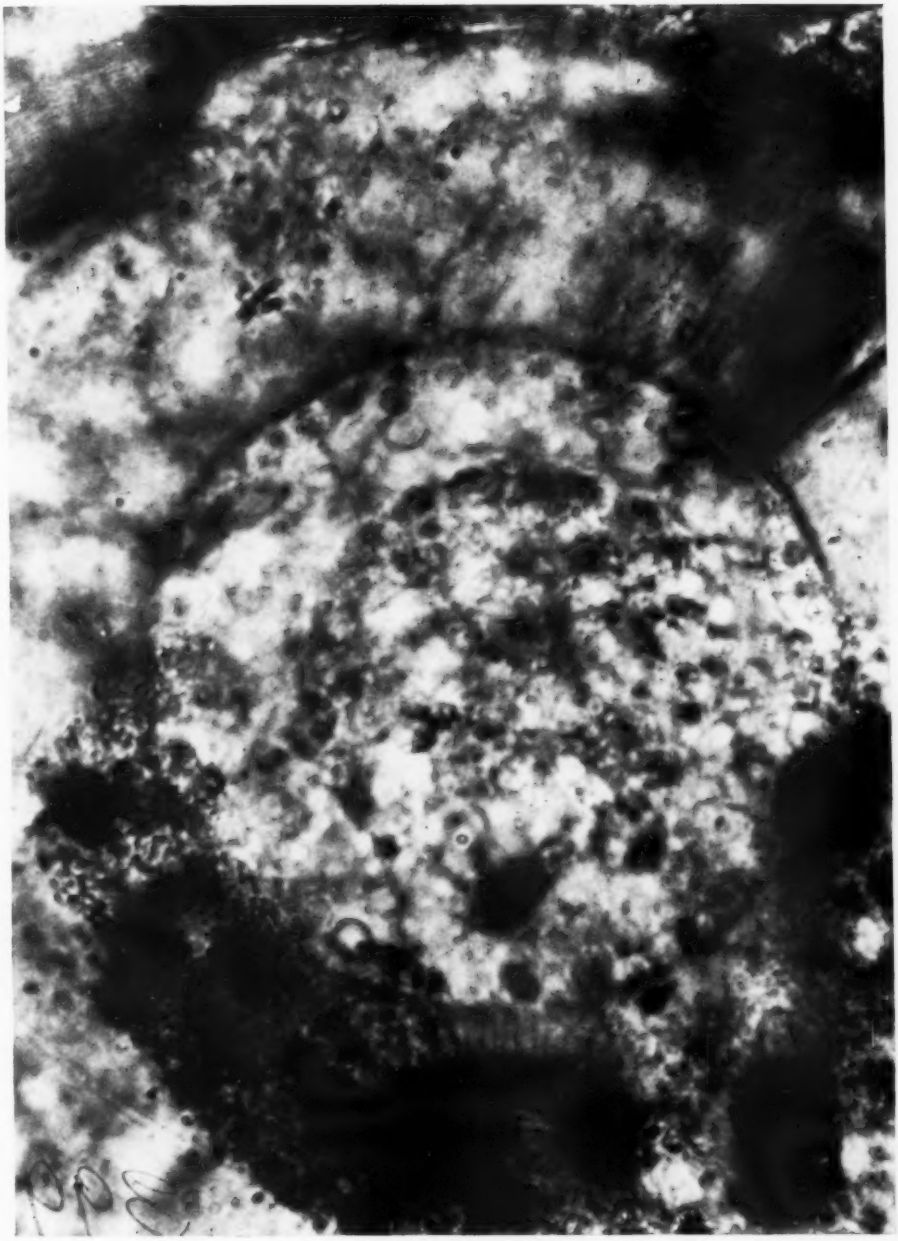


PLATE 123

FIGURE 16.—Microinjection of X-rayed cytoplasm. Host cell has received injection 1 hour previously. Note pyknotic central mass in nucleus and heavy spherical flocculation of cytoplasm. Compare with Plates 121, 122, and 124. *Triturus viridescens*. × 920.

PLATE 124

FIGURE 17.—Same cell as in Plate 123, 2 hours post injection. Cytoplasmic material from animal irradiated with 3,000 r, 7 days previously. Note advanced central nuclear body (C. N. B.) and swollen vacuolated nucleoli. Compare with Figures 4, *B*, 5, *B*, and 14. *Triturus viridescens*. × 760.

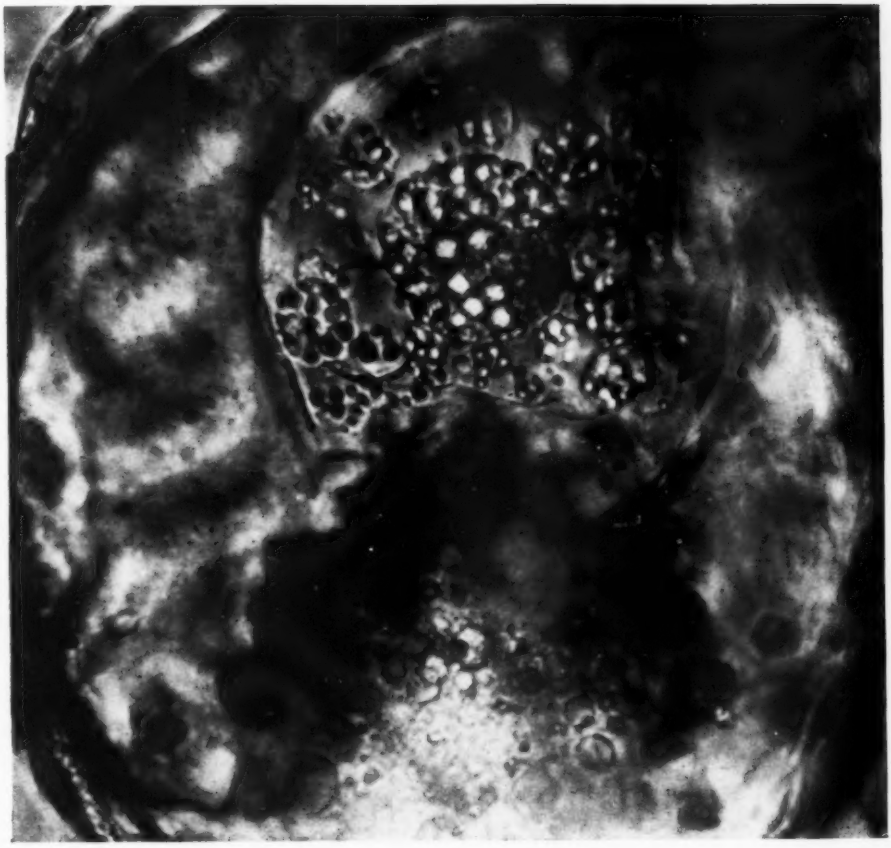
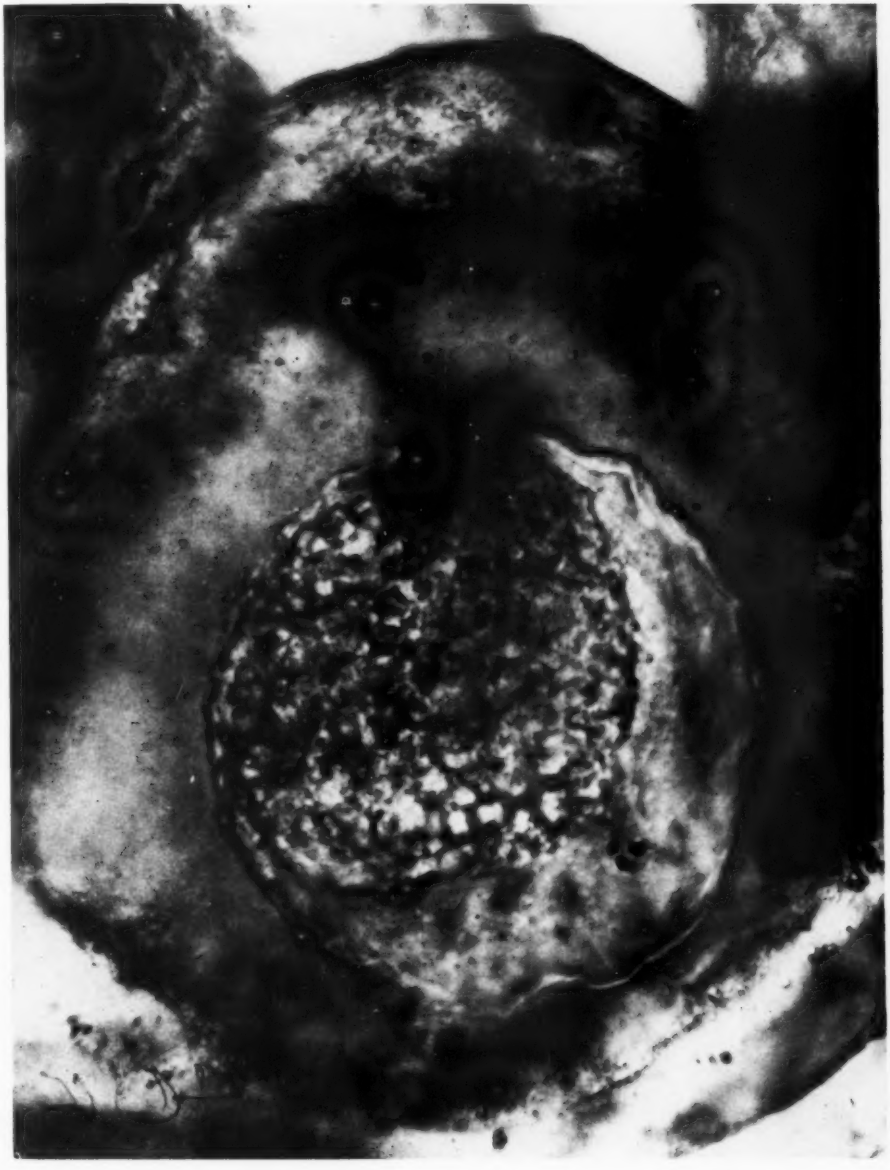


PLATE 125

FIGURE 18.—Effect of long continued exposure to cold (50° C.) following irradiation. Note flocculating cytoplasm shrinking away from cell wall. Inside nucleus similar process taking place. However, pyknosis is not yet apparent. Most damage is still in cytoplasm. *Triturus viridescens*, 3,000 r, 21 days postirradiation. × 780.



ABSTRACTS OF ARTICLES PUBLISHED BY MEMBERS OF THE STAFF OF THE NATIONAL CANCER INSTITUTE

METHYL-BIS(BETA-CHLOROETHYL)AMINE IN LARGE DOSES IN THE TREATMENT OF NEOPLASTIC DISEASES. *H. R. Bierman, M. B. Shimkin, S. R. Mettier, J. Weaver, Wilbur C. Berry,* and *Samuel P. Wise III.* California Medicine 71 (2) August (1949).

Sixty-seven patients with neoplastic diseases were treated with 151 courses of methyl-bis(beta-chloroethyl)amine hydrochloride (HN_2). Seventy-seven of the courses consisted of single injections of 0.2 to 0.4 mg. per kilogram of body weight, and 35 courses were given as single injections of 0.6 mg. per kilogram of body weight.

Twenty-three patients with Hodgkin's disease were treated. Remissions averaged approximately 3 months in 13 patients who were in good or fair general physical condition and 1.5 months in 11 patients who were in poor or moribund condition; 1 of the 11 did not respond to the therapy.

Fifteen patients with lymphosarcoma were treated. Remissions averaged between 1 and 2 months in 4 patients who were in good or fair general physical condition. Of the remaining 11 patients, 2 showed no response, and the longest remission among the remaining 9 was approximately 40 days.

Satisfactory remissions of 1 to 3 months were obtained in 4 patients with mycosis fungoides treated with single courses of 0.3 mg. per kilogram of body weight. Serious toxic reactions were observed in 6 patients, 4 of whom died. In 5 of the 6 instances the reactions consisted of pancytopenia and hemorrhagic diathesis. All these patients were in poor general or hematologic status before therapy.

In general, large single doses of HN_2 were neither more nor less effective than the 4- to 6-day course usually employed with this agent. Combination of the administration of HN_2 with artificial hyperpyrexia or with concurrent courses of pteroylglutamic conjugates did not enhance the therapeutic effects of the agent.

A METHOD FOR ESTIMATING VOLUME-SURFACE RATIOS. *Harold W. Chalkley, Jerome Cornfield,* and *Helen Park.* Science 110: 295–297 (1949).

A method is developed for estimating volume-surface ratios of morphological components in sectioned material by throwing a line at random on the sections. An empirical verification of the method by Chalkley of sectioned material of known dimensions is discussed.

ENZYMATIC HYDROLYSIS OF SATURATED AND UNSATURATED TRIPEPTIDES. *Paul J. Fodor, Vincent E. Price,* and *Jesse P. Greenstein.* J. Biol. Chem. 180: 193–208 (1949).

A considerable number of dipeptides and tripeptides, some of them containing all saturated and some containing unsaturated peptide bonds, were incubated with aqueous extracts of hog or rat kidney under conditions whereby nearly the maximum hydrolysis of the susceptible bonds was achieved.

Like chloroacetyldehydroalanine, chloroacetyl-L-alanine, chloraocetyl-L-phenylalanine, and chloroacetyl-L-leucine, the tripeptides, chloroacetylglycyldehydroalanine, chloroacetylglycyl-L-alanine, chloroacetylglycyl-L-phenylalanine, and chloroacetylglycyl-L-leucine are completely hydrolyzed. Again, like chloroacetyldehydrophenylalanine, chloroacetyl-D-alanine, chloroacetyl-D-phenylalanine, and chloroacetyl-D-leucine, the tripeptides, chloroacetylglycyldehydrophenylalanine, chloroacetyl-

glycyl-D-alanine, etc., are practically completely resistant to enzymatic action of hog kidney. Inasmuch as the dipeptides themselves, such as glycyldehydrophenylalanine, glycyl-D-alanine, are readily hydrolyzed, it would appear that the susceptibility of the chloroacetylated dipeptides and dehydropeptides was governed largely by the nature of the acyl and terminal residues.

The pH-activity curve for chloroacetyldehydroalanine shows a broad optimal zone between pH 6.5 and 9. That for chloroacetyl-L-alanine has a relatively sharp optimum at pH 7.2. The pH-activity curves for chloroacetylglycyldehydroalanine, glycyldehydroalanine, chloroacetylglycyl-L-alanine, glycyl-L-alanine, and chloroacetyl-DL-alanylglycine, all show an optimum at about pH 8.0.

Chloroacetyl-DL-alanylglycine, chloroacetyl-DL-leucylglycine, and chloroacetyl-DL-phenylalanylglycine are half hydrolyzed, and it is presumed that it is the L-moities of these racemic tripeptides which are susceptible. Chloroacetyldehydrophenylalanylglycine is completely resistant.

The glycylglycylated DL-amino acids and glycylglycylated dehydroamino acids are completely hydrolyzed, probably through the primary action of aminopeptidase on the susceptible aminoacyl bond. Glycylsarcosyldehydroalanine is slowly hydrolyzed, in accord with the low degree of susceptibility of the glycylsarcosine bond. The isomeric tripeptides, such as glycyl-DL-alanylglycine, glycyl-DL-phenylalanylglycine, and glycyl-DL-leucylglycine are hydrolyzed at three or close to three bonds, instead of the theoretical four. The reason for this presumably lies in the relative resistance of the bond between the D-amino acid and the terminal glycine residue. Such dipeptides as D-alanylglycine are hydrolyzed extremely slowly. Glycyldehydrophenylalanylglycine is completely resistant, a phenomenon which at once sets off the dehydropeptidase from the peptidases which act upon the analogous saturated peptides.

Approximate rate studies were made with several types of substrates. The aminoacylated substrates are hydrolyzed at a faster rate than the corresponding chloroacetylated substrates, whether dipeptides or tripeptides, or containing saturated or unsaturated bonds. The peptides are in general hydrolyzed at a faster rate than the dehydropeptides, except for glycyl-D-alanine which is cleared at a slower rate than glycyldehydroalanine. Hydrolysis of chloroacetyl-DL-phenylalanylglycine and of chloroacetyl-DL-alanylglycine was considerably faster than that, respectively, of the isomeric substrates, chloroacetylglycyl-DL-phenylalanine and chloroacetylglycyl-DL-alanine. It is suggested that different enzyme entities act upon di- and tripeptides.

EFFECT OF ANIONS ON THE NON-ENZYMATIC DESAMIDATION OF GLUTAMINE. *James B. Gilbert, Vincent E. Price,* and *Jesse P. Greenstein.* J. Biol. Chem. 180: 209–218 (1949).

The effect of various anions on the desamidation of glutamine in veronal-buffered solution has been studied and compared with the effect of the same ions on the enzymatic desamidation of this substrate in rat tissue aqueous extracts.

The nonenzymatic reaction which leads to the formation of pyrrolidonecarboxylic acid and ammonia is accelerated by phosphate, phosphite, arsenate, methylarsenate, borate, and bicarbonate and is not accelerated by sulfate, nitrate, acetate, chloride, or pyruvate at equivalent concentrations. Chloroacetylglutamine is not affected whether phosphate is or is not present. The enzymatic reaction which leads to the formation of glutamic acid and ammonia is accelerated by phosphate, phosphite, arsenate, methylarsenate, sulfate, and pyruvate and is not accelerated by borate, bicarbonate, nitrate, acetate, or chloride at equivalent concentrations.

The desamidation of glutamine in tissue extracts occurs at an optimum at the same pH whether accelerating salt has been added or not. The accelerating effect of phosphate on the nonenzymatic desamidation of glutamine increases with increasing pH, and decreases with decreasing pH.

The several differences between the phenomena of enzymatic and nonenzymatic desamidation reactions suggest that the latter occur by different mechanisms.

RESOLUTION OF RACEMIC PHENYLALANINE, TYROSINE, AND TRYPTOPHAN. *James B. Gilbert, Vincent E. Price,* and *Jesse P. Greenstein.* J. Biol. Chem. 180: 473–478 (1949).

Racemic phenylalanine, tyrosine, and tryptophan have been readily resolved into their optical isomers by subjecting their N-chloroacetylated derivatives to asymmetric hydrolysis by a purified hog pancreas carboxypeptidase preparation.

In contradistinction to the susceptible chloroacetyl derivatives of phenylalanine and of tryptophan, the corresponding acetyl derivatives are very resistant to the action of pancreatic carboxypeptidase.

THE RESOLUTION OF SEVERAL RACEMIC AMINO ACIDS. *Vincent E. Price, James B. Gilbert,* and *Jesse P. Greenstein.* J. Biol. Chem. 179: 1169–1182 (1949).

The method of resolving N-acylated racemic amino acids by means of asymmetric enzymatic hydrolysis of the L-acylated isomer, previously described for the case of alanine, has been extended to include the resolution of racemic methionine, valine, threonine, isoleucine, serine, leucine, and aspartic and glutamic acids.

RADIOACTIVE SODIUM PERMEABILITY AND EXCHANGE IN FROG EGGS. *Philip H. Abelson* (Dept. of Terrestrial Magnetism, Carnegie Institution) and *William R. Duryee* (National Cancer Institute). Biol. Bull. 96: 205–217 (1949).

A new method is described to distinguish between life and death by means of permeability to Na^{24}. By exposing excised live ovarian eggs of *Rana pipiens* to radioactive Ringer solution, it was found that an average egg would exchange 2.6×10^{-7} gm. Na after 1 hour. However, dead cells exchanged up to seven times this amount in only 10 minutes. A new method of making radioautographs of single cells frozen in liquid air showed that, after 30 minutes, the Na was evenly distributed throughout the cytoplasm, but at 2, 4, 6, and 8 minutes of exposure "ring" pictures of increasing depth were found. Calculations based on Geiger counter measurements as well as on the radioautographs gave a value of 2.6×10^{-7} cm^2/sec. for the diffusion constant of Na in cytoplasm. Since dead eggs concentrated Na^{24} up to 40×10^{-7} gm. Na per cell (above the level of 25.8×10^{-7} gm. Na per egg as determined chemically), it was concluded that two different types of binding limit the diffusability of sodium within the cell. Only 12 percent of the normal sodium was readily exchangeable. The remainder exchanged very slowly.

THE MAXIMUM EFFICIENCY OF PHOTOSYNTHESIS: A REDISCOVERY. *Dean Burk, Sterling Hendricks, Mitchell Korzenovsky, Victor Schocken,* and *Otto Warburg.* Science 110: 225–229 (1949).

The efficiency of the energy transformation "chemical energy gained/light energy absorbed" in photosynthesis by *Chlorella pyrenoidosa* was found to attain a maximum of at least 65–85 percent (3–4 quanta of red light per molecule of O_2 produced or CO_2 consumed) under readily obtainable, reproducible, and demonstrable experimental conditions at pH 5. These high efficiencies were measured not only with low light intensities below the compensation point and for short periods of time (minutes) but also with light intensities 5 to 10 times the respiration-compensation intensity in experiments lasting many hours. Allowance for respiration during photosynthesis was made unambiguously or eliminated altogether in calculating the photosynthetic yield. In carbonate buffer (pH 9) the efficiency was only one-half to one-third as great as in culture medium at pH 5. The assimilatory quotient (CO_2 consumed/O_2 produced) averaged -1.08 ± 0.25, without evidence of the so-called CO_2 outburst or inburst. To ensure the results reported it is desirable that the Chlorella cultures be rapidly aerated with 5 percent CO_2 in air, frequently transferred into fresh medium every few days, and heavily inoculated with 100–200 cu mm. cells per 200 cc. medium,

and in the efficiency measurements provision must be made for maintenance of the dark (Blackman) reaction at full rate by adequate shaking.

DOES LIGHT INHIBIT THE RESPIRATION OF GREEN CELLS? *Otto Warburg, Dean Burk, Victor Schocken, Mitchell Korzenovsky,* and *Sterling B. Hendricks.* Arch. Biochem. 23: 230–233 (1949).

The oxygen consumption of green cells may be decreased by illumination. The light intensity at which the oxygen exchange of a given culture becomes zero may be termed "compensating." Experiments show conclusively that red light (630–660μ) does not inhibit Chlorella respiration per se when light intensities are employed that yield high photosynthetic efficiencies. When light compensates respiration, it does so by the independent process of photosynthesis, the gas exchange of which happens to be the opposite of that of respiration. The same high quantum efficiencies of 3–5 quanta per O_2 produced are obtained both below the compensation point and up to intensities at least five times the compensating intensity. That is, one molecule of oxygen developed above the compensation point, or one less molecule of oxygen consumed below the compensation point, as the result of light action, represents the same gain in chemical energy. All theories of light action should be in harmony with this now-established thermodynamic fact.

CORRELATED DECREASES IN GLYCOLYSIS AND INOCULATION TAKE OF IN VITRO INDUCED SARCOMA CELLS UPON PROLONGED SUBCULTURE. *Dean Burk, Wilton R. Earle, Marie L. Hesselbach, Clara E. Fischer, Emma Shelton,* and *Edward L. Schilling.* Cancer Research 9: 558–559 (1949).

Marked changes in metabolism and percentage-effective inoculation have been noted among series of mouse tumor transplants newly derived from strains of mouse fibroblasts long-continued in tissue culture. The L, D, and H strains of Earle sarcomas, when newly derived from tissue cultures, showed considerably reduced glycolysis in the second to sixteenth transplant generations, as compared with these tumor strains when initiated several years before, and also to L and D strains in about the fifty-fifth to sixty-fifth transplant generation. The decrease in glycolysis was about 25 percent at pH 7.3, the usual pH of measurement, but this comparative decrease was greatly enhanced when the measurements were made at pH 6.3, where the decrease attained 80 to 90 percent. A correlated decrease in malignancy of these strains was also indicated by a notable reduction in the percentage of takes from tissue culture cells inoculated into mice. Between 1942 and 1945, the percentage of takes in strains D, H, and L fell from 86, 74, and 70 to 50, 8, and 2 percent, respectively.

Glycolysis of various Earle strain sarcoma tissue slices was found to be almost entirely eliminated within a few hours by exposure to pH 6.3. Return to pH 7.3, after increasing times of exposure to pH 6.3, resulted in decreased ability to recover, depending upon strain. Embryonic chick tissues had considerably less ability to withstand low pH (or recover from exposure thereto) than any of the tumor tissues studied. Anaerobic glycolysis was found (Earle strain H) to require neither CO_2 nor bicarbonate at any measurable concentration, and proceeded essentially as rapidly in phosphate medium as in CO_2-bicarbonate medium, comparable pH values being maintained.

PROGESTERONE AND TESTOSTERONE REVERSAL OF DIETHYLSTILBESTROL INHIBITION OF SUCCINOXIDASE OF TUMOR MITOCHONDRIAL ELEMENTS. *Kent Wight* and *Dean Burk.* Fed. Proc. 8: 265 (1949).

It has been found that certain hormones act competitively on the succinoxidase system of the mitochondrial elements of the S91 and Harding-Passey melanomas grown in dba and C mice respectively. The mitochondrial elements (microscopically visible cytoplasmic particulates, mostly melanized; see Woods, et al. J. Nat. Cancer Inst. 9: 311–323 (1949)) were obtained by centrifugation at 10° C. of saline, glass

wool-filtered tumor homogenates at 10,000 g for 30 minutes, after preliminary centrifugation at 25 g for 10 minutes to eliminate nuclei and heavy phagocytized cell material. Oxygen consumption was measured manometrically after addition of 0.05M phosphate (pH 7), 0.00001M cytochrome C, and 0.02M succinate. Diethylstilbestrol, at a concentration of ~135γ/ml. (⇔ 0.0005M), inhibited particulate oxygen consumption 60 to 75 percent. This inhibition was markedly reduced or entirely eliminated by the simultaneous addition of ~125γ/ml. (⇔ 0.0002M) progesterone or testosterone. Progesterone alone had no effect on oxygen consumption. The S91 and Harding-Passey tumor particulates behaved similarly except that the normal Q_{O_2} values (ml. O_2 consumed/mg./dry wt./hour) were about 5 times as great in the case of the former (Q_{O_2} of 10–20 compared to 2–5) and that testosterone alone sometimes inhibited the latter. Essentially no succinoxidase activity was shown by the initial nuclei fractions or the final supernatants. Diethylstilbestrol also markedly inhibited or eliminated oxygen consumption by mitochondrial elements from nonmelanoma tumors such as C3HBA breast carcinoma and sarcoma 37 grown in C3H and C mice respectively. The foregoing observations on sex hormone antagonisms at the mitochondrial enzyme level may offer some basis of mechanism for the numerous similar antagonisms noted at organ and body levels.

CERTAIN EFFECTS OF ANTIBIOTICS ON ANIMAL TISSUE HOMOGENATES AND SUBCELLULAR ELEMENTS. *Kent Wight* and *Dean Burk*. J. Clin. Invest. 28: 1041–1042 (1949).

These studies were carried out using aerobic methylene blue reduction and Warburg techniques. For the methylene blue reduction studies, mouse livers were homogenized with a mortar and pestle in 0.01M phosphate. The substrates were malate, pyruvate, acetate, lactate, alcohol, oxalacetate, fumarate, and citrate at 0.01M and in each case 4 p. p. m. methylene blue. Purified penichromin, a yellow component of crude penicillin, increased the reduction time 100 percent above that of the control. With malate present, the effect was even more striking. Penicillin and streptomycin increased the reduction time as much as 50 percent in concentrations of 1,000 p. p. m. or higher. The same effects were observed with acetate and in the case of streptomycin also with ethyl alcohol. For pyruvate none of the antibiotics inhibited methylene blue reduction. Other antibiotics studied were subtilin and siderophilin.

The tumors used were the S91A grown on C mice and the C3HBA on C3H mice. The time of reduction of methylene blue by the S91A tumor fractions was one-fifth the time required by liver homogenates. This was further affected by penichromin (>1,000 p. p. m.) to the extent of 100 percent.

The particulate fractions were prepared by homogenizing the tumors in 0.85 percent saline, filtering through glass wool, and centrifuging at 26 g for separation of the nuclei followed by 9,000 g for the mitochondria.

In the case of the S91A tumor homogenates, the manometric studies showed inhibition of succinate oxidation by penichromin at 500 p. p. m. of 50 percent and no effect by actidione, aspergillic acid, or subtilin.

For the mitochondrial fraction using the C3HBA tumor in the presence of cobalt at 50 and 20 p. p. m., the $Q_{O_2}^{N}$ of 4.6 was decreased to 1.4 and 3.1 respectively. Similar results were obtained with penichromin.

Thus some antibiotics have a striking effect on the enzymes of animal tissues at the subcellular level while some do not except at very high concentrations.

THE EFFECT OF STREPTOMYCIN ON DEAMINATION AND OXYGEN CONSUMPTION BY RESTING CELLS OF E. COLI. *Kent Wight* and *Dean Burk*. Fed. Proc 7: 199 (1948).

Warburg technique studies were carried out on the mode of action of streptomycin on resting cells of *E. Coli*, strain NIH 119, grown 16 to 24 hours in nutrient broth at 37° C., centrifuged, washed, and resuspended in 0.50M phosphate at pH 7.4, and then studied 4 to 5 hours at 37° C., with and without addition of 10 to 20 p. p. m. streptomycin, 0.012M amino acid, or 0.007M nonnitrogenous organic acid.

Anaerobically, in N_2, the washed, resting *E. coli* cells deaminated aspartic acid and serine, but not threonine, alanine, or histidine. Streptomycin inhibited this ammonia production up to 50 percent in the case of aspartate and 35 percent with serine. No CO_2 production was detected with any of the five amino acids, even in the presence of 0.01–0.02M semicarbazide. Oxaloacetate and pyruvate, but not acetate, yielded CO_2, without inhibition by streptomycin, however.

In air, ammonia production from aspartate and serine was somewhat greater than in N_2, and the percentage inhibition by streptomycin was somewhat less (20–30 percent). At the same time, oxygen consumption (Q_{O_2}=ca. 30) was decreased about 50 percent by the fourth hour by streptomycin. Threonine and alanine gave the same oxygen consumption as aspartate, but less NH_3 production, and no inhibition of either process by streptomycin. The Q_{O_2} with fumarate was decreased by streptomycin from 40 to 25 and with oxaloacetate from 90 to 50. However, streptomycin did not inhibit either the aerobic or anaerobic assimilation of ammonia when added with fumarate, the presumed end-product of aspartate deamination.

COBALTODIHISTIDINE AND RELATED COMPOUNDS AND THEIR SIGNIFICANCE IN OXYGENATION OF METALLOPROTEINS. *Dean Burk, John Hearon, Silvio Fiala, Arthur L. Schade,* and *Hilton Levy*. Abstracts, 113th meeting, Am. Chem. Soc., pp. 34C–35C (1948).

The ability to combine with oxygen gas reversibly and stably under physiological conditions, demonstrated so far for hemoglobin, myoglobin, hemocyanin, and oxidases like *Atmungsferment*, all Fe- or Cu-proteins of high molecular weight, has also been found with various simple cobaltous amino acid complexes, including cobaltous compounds of histidine, 1-methylhistidine, 3-methylhistidine, benzylhistidine, dibenzylhistidine, aspartylhistidine, carnosine, anserine, histamine, lysine, arginine, tryptophane, proline, ornithine, serine, asparagine, glutamine, glutamate, glycine, and glycylglycine (though not with e. g., cysteine, methionine, aspartate, putrescin, or β- or α-alanine).

The oxygen affinity constants of the reversibly oxygenated complexes may vary by several orders of magnitude, with different amino acids. Thus, with 1-methylhistidine and dibenzylhistidine the equilibrium constants at 23° C. were 625 and 133 times smaller than with histidine, where $K_{O_2}=((Hist_2\,Co^{++})_2O_2)/(Hist_2Co^{++})^2(O_2\,aq)=6\times10^8$ liters^2 per moles.^{2} These differences are probably due mainly to steric hindrance, since with 3-methylhistidine and (3-)benzylhistidine the equilibrium constants were merely 0.42 and 2 times as great as with histidine. The absence of the carboxyl group in histamine yielded an eight-fold greater constant of oxygenation with this compound; on the other hand, the equilibrium constant for simple (unoxygenated) chelation between Co^{++} and histamine anion was considerably smaller than with Co^{++} and histidine anion ($K=(Hist^-{}_2Co^{++})/(Hist^-)^2(Co^{++})$), corresponding to a difference in stability of 4.1 Kcal per mole, due presumably to absence of electrostatic interaction between Co^{++} and $-COO^-$ in histamine.

The reversibly oxygenated complexes, which are yellow-brown, are transformed spontaneously and irreversibly into pink forms, with velocities that vary enormously, requiring minutes, hours, or days for completion at concentrations of 0.001 to 0.1M and temperatures of 20–45° C; oxy-bis (cobaltodihistidine) and oxy-bis(cobaltodihistamine) are ordinarily the stablest by far. The irreversible reactions may or may not involve additional uptake of O_2, production of $CO_2(HCO_3^-)$, or production of OH^-, depending upon conditions, but as yet no evident oxidation of Co^{++} to Co^{+++}.

It is possible to ascribe the stabilization of the cobaltous state in oxy-bis(cobaltodihistidine) to the pairing of a single electron of Co^{++} in the $4p$ orbital with a single electron in O_2 made available by rupturing one of the two 3-electron bonds, thereby yielding a distribution of $3d^{10}4s^{2}4p^{6}$ (krypton configuration). A change in electronic structure of the O_2 molecule and a loss of paramagnetism, upon oxygenation of cobaltodihistidine, are likewise observed upon oxygenation of the iron-protein hemoglobin. Cobaltodihistidine is, of course, more comparable to the Cu-protein hemocyanin, rather than hemoglobin, in regard to its lack of combination with CO, and its metal: O_2 ratio of 2:1.

EXCERPTA MEDICA

INDEXES AND ABSTRACTS OF MEDICAL LITERATURE[1]

. . . In 1947 the government of the Netherlands subsidized the publication of a monthly abstracting service of articles from every available medical journal in the world under the title *Excerpta Medica*. This is published in fifteen sections and covers the whole field of clinical and experimental medicine. Many hundreds of abstracters throughout the world cooperate in this project. The organization, which is under the general editorship of M. W. Woerdeman, professor of anatomy and embryology at the University of Amsterdam, is growing in scope. Already many of the sections are in process of developing their third volume. Recently the *Excerpta Medica* was reorganized on a not for profit basis; a conference to be held by UNESCO in April will determine the extent to which that agency can support a further development of the various abstract services now available. At present physicians will find the *Excerpta Medica* the most comprehensive and prompt abstracting of the medical literature of the world thus far available . . .

More recently the editor of THE JOURNAL has been associated by action of the Board of Trustees with the publication of *Excerpta Medica*. This association is designed to bring about coordination of the type so badly needed. Particularly in view is a possible coordination between abstracting services and indexing. As soon as the publication of the Quarterly Cumulative Index Medicus is brought up to date, the editors hope to indicate in the Index the references to abstracts that will be easily available in *Excerpta Medica*. This should enhance the value of both the Index and *Excerpta Medica* . . .

Medical periodicals exist by the support of physicians and medical institutions. The publication of the Index Medicus has been carried on by the American Medical Association for many years with a considerable contribution from the funds of the Association. *Excerpta Medica* likewise is still published at a loss which could be largely overcome if enough physicians gave their support either to the total publication or to the individual sections in which they happen to be interested. Since medical progress is dependent on the rapid dissemination of medical information, this important project merits support.

[1] Reprinted from the J. A. M. A. 139: 1080–1081 (1949).

ANNOUNCEMENT

Fifth International Cancer Congress

Under the eminent patronage of the President of the French Republic and under the auspices of the International Union Against Cancer

PARIS, July 15th–22th 1950
Congress Seat: LA SORBONNE
47, rue des Écoles

While awaiting the opening of Congress address all correspondence to the Union Internationále contre le Cancer 6, Avenue Marceau, Paris (8e)
Telephone: ELYsées 92–80

President of Congress, Prof. A. Lacassagne.
General Secretary, Prof. V. Le Lorier.
Adjoint General Secretary, Dr. P. F. Denoix.
Treasurer, B. H. Flurscheim.

Program Committee

President, Prof. Charles Oberling

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GENERAL ORGANIZATION

The Fifth International Cancer Congress will be held in Paris during the month of July 1950. It will coincide with the commemorative manifestations of the fiftieth anniversary of the discovery of Radium by PIERRE and MARIE CURIE. But the Congress proper will last from Monday the 17th to Friday the 21th of July.

The Congress seat will be at the SORBONNE. All sessions of the five day meeting will take place in different amphitheaters of this establishment from 9 o'clock in the morning to noon, and from 2 to 5 o'clock in the afternoon. An exhibition reuniting the documents relating to research, statistics, organization of centers of treatment, equipment and social struggle, will be held at the same time in the SORBONNE.

The communications addressed by the Congressmen will be distributed in the three following branches:

1° Biology and Experimentation.
2° Pathology, Clinic and Therapy.
3° Social Struggle.

Sections will be constituted in each of these branches. The number of meetings attributed to the sections will depend on the number of communications inscribed.

The organization of this Congress has been discussed at the course of the General Assembly of the International Union against Cancer which was held in Paris, from the 18th to the 23rd of July 1949. It has been decided that in each of the considered branches, a certain number of symposia would be organized, which will be held each morning and each afternoon of the 5-day meeting, in a special amphitheater reserved for this purpose. The symposia of Biology and Social Struggle will take place in the morning; those of Pathology, Clinic and Therapy in the afternoon.

In order to be admitted as a member of Congress, it is necessary to belong to a well known scientific or social association, or to be recommended by such an association.

All participants regularly inscribed can present at least one communication. The exact title of the treated subject and a summary not more than 250 words, will have to reach the Secretary's Office, 6, avenue Marceau, Paris (8e), no later than the first of April 1950, for want of, the communication will not figure in the program. The duration of the report will be limited to 10 minutes. Consignment of all communications will have to be preceded or accompanied by the detachable leaflet B,* and addressed to the Secretary's Office before the first of April 1950.

During the time the Congress convenes, visits will be organized in the different Centers of research and treatment of cancer in Paris and in the parisian neighborhood.

A program of amusement and receptions for congressmen and their families is also provided.

An excursion to Reims will take place on Saturday 22nd of July. Congressmen who desire to participate are requested to fill in the detachable leaflet D,* and to address it to the Secretary's Office before the first of April 1950.

The registrations at Congress are received from this moment at the seat of the International Union against Cancer, 6, avenue Marceau, Paris. The detachable leaflet A,* will have to accompany each request for registration.

The amount of the fee is as follows: titular active members $10; junior members (active members less than 30 years on the first of January 1950), $5; associate members, $3.

The dues will have to reach the Secretary's Office before the first of April 1950. After this date the amount will be raised to: Active members, $13; junior members, $7; associate members, $4.

Congressional members who desire to retain reservation for trips and hotel spaces are requested to apply to Agency Cook & Son, or to the Pullman International Co., by sending to them the detachable leaflet C*.

Members of Cancer Congress desiring to return to the Sixth International Congress of Radiology, which will be held the following week in London, are requested to notify the Secretary's Office by connecting the detachable leaflet E*, in order to make possible the eventual organization of the transportation in groups of the Congressmen.

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*[*Editor's Note.*] Obtainable through Dr. Charles Hooker, Secretary, American Association for Cancer Research.